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Page No.	Date of Issue	Date of Return
116	21-9-53	24.9.53
31	6-6-54	16.6.54.
53	9. 11. 55	18-11-55
190	20-1-56	8/4/57
41	17-1-57	29.4.57
5	1 JUL 1958	3/6/58
48	29 NOV 1958	31 OCT 1958

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STANDARD METHODS

FOR

THE EXAMINATION OF DAIRY PRODUCTS

MICROBIOLOGICAL, BIOASSAY
AND CHEMICAL

Eighth Edition

Microbiological and Bioassay Methods formulated by Committees of the American Public Health Association and approved for publication by the American Public Health Association, March 20, 1941.

Chemical Methods compiled by the Committee on Editing Methods of Analysis of the Association of Official Agricultural Chemists and approved for publication by that Association at its Annual Meeting in Washington, November 7, 1940.

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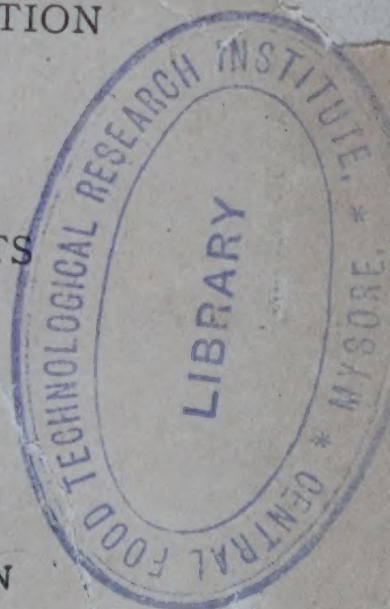
OFFICIAL AGRICULTURAL CHEMISTS

Box 290, Pennsylvania Avenue Station

Washington, D. C.

Published by the
AMERICAN PUBLIC HEALTH ASSOCIATION

1941



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Eighth Edition
Third Printing

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Standard methods..

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PREFACE TO THE EIGHTH EDITION

THE Eighth Edition of *Standard Methods for the Examination of Dairy Products* includes all of the methods outlined in the Seventh Edition of this report with the addition of much entirely new material in Part II, Methods for the Examination of Frozen Desserts. The latter section includes methods for the examination of plain ice cream and other frozen desserts, together with methods for the examination of common ingredients of frozen desserts, such as powdered milk, evaporated milk, condensed milk, dry milk, coloring solutions, flavoring extracts, fruits, nuts, sweetening agents, and egg products. Part I covering Methods for the Examination of Milk, Cream and Butter has been revised and brought up to date. The section which outlines methods for determining the sterility of bottles, cans, and other dairy utensils has been enlarged to include methods useful for determining the sterility of sanitary closures and caps, paper containers and special ice cream and other frozen desserts equipment. New methods for the examination of butter have been included.

Wherever methods are given that have not previously been widely distributed to interested members of the Association and revised in the light of comments received, they are indicated as Tentative.

The Microbiological Methods given in this new edition have been prepared under the direction of the following Committees of the American Public Health Association: Standard Methods for the Examination of Dairy Products: R. S. Breed, Chairman, Mac H. McCrady, A. H. Robertson, W. S. Frisbie, R. V. Stone, A. J. Slack, S. R. Damon, and W. D. Tiedeman. This committee has been assisted by the following group of Associate Referees: F. W. Fabian, E. H. Parfitt, G. J. Hucker, H. R. Thornton, C. C. Carson, I. F. Huddleson, W. A. Hagan, and J. H. Shrader. Committee on Standard Methods for Analyzing Frozen Desserts and Ingredients: F. Lee Mickle, Chairman, F. W. Fabian, J. H. Shrader, A. H. Robertson, James Gibbard, and M. E. Parker, assisted by the following group of Associate Referees: P. S. Prickett, P. A. Downs, M. J. Prucha, H. H. Hall, Roy Schneider, H. H. Sommer, O. A. Ghiggoile, W. H. Martin, George Jaggard, P. H. Tracy, F. L. Hart, A. C.

Dahlberg, T. C. Downey, A. C. Fay, M. P. Horwood, A. G. Lochhead, and P. S. Lucas.

The Bioassay Section (Part III) of the Report which remains without change was prepared by the Committee on Assay of Foods: H. T. Scott, Chairman, M. Ant, F. D. Baird, P. L. Day, C. A. Elvehjem, C. R. Fellers, E. M. Nelson, and R. W. Pilcher. Their work has been carried out in coöperation with the individuals responsible for the methods given in the U. S. Pharmacopoeia and in the Methods of Analysis of the Association of Official Agricultural Chemists.

This work is under the general supervision of the Coördinating Committee of the Laboratory Section: A. P. Hitchens, Chairman, R. S. Breed, W. D. Stovall, F. Lee Mickle, R. A. Kelser, E. S. Robinson, W. L. Mallman, J. Gibbard, and E. K. Kline; the Coördinating Committee of the Food and Nutrition Section: F. C. Blanck, Chairman, F. W. Fabian, H. E. Goresline, G. J. Hucker, M. J. Mack, B. E. Proctor, and H. T. Scott; and of the Committee on Research and Standards of the Association: K. F. Maxcy, Chairman, G. W. Anderson, Margaret G. Arnstein, R. A. Bolt, H. L. Dunn, Haven Emerson, G. Fair, W. S. Frisbie, I. V. Hiscock, A. P. Hitchens, J. F. Norton, G. C. Ruhland, T. F. Sellers, L. R. Thompson, W. D. Tiedeman, A. Wolman, and R. M. Atwater.

The Chemical Section (Part IV) of the Report has been prepared by a Special Committee of the Association of Official Agricultural Chemists consisting of E. M. Bailey, Chairman, G. G. Frary, and W. S. Frisbie. The latter Association is solely responsible for the methods given in the Chemical Section.

Methods for determining phosphatase are given in an Appendix as in the Seventh Edition. Two of these are now recognized as Tentative Methods by the Association of Official Agricultural Chemists.

Again the American Public Health Association wishes to express its appreciation to all who have contributed toward the new material or improvements introduced with this edition.

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HISTORICAL INTRODUCTION

It is interesting to note various milestones in the development of the use of laboratory methods for controlling the sanitary quality of market milk. Probably the first publication that should be noted is one by Sedgwick and Batchelder¹ in 1892 in which the results of their bacteriological examination of Boston milk brought to the public a realization of the importance of dairy sanitation. The beginnings of certified milk² in 1893 by Dr. Coit and Stephen Francisco at Montclair, N. J., also involved the use of laboratory methods for controlling the number of bacteria present in milk, and the city of Montclair is said to have been the first city in America to undertake regular bacteriological examinations of its milk supply. The studies of the sources of bacteria in milk by H. W. Conn³ at Middletown, Conn., as early as 1889 also had their stimulating effect. These were followed by similar studies by H. L. Russell⁴ at Madison, Wis., in 1894-95. The first positive action in New York City came in 1896 when the Board of Health established a permit system with attendant rules and regulations governing the sale of milk. Its right to make these regulations was challenged in the courts and the fight was carried to the United States Supreme Court, which rendered a decision in 1905 supporting the position taken by the board. From that time the right of officially constituted Boards of Health to control the sanitary quality of municipal milk supplies has never been successfully opposed.

The publication by W. H. Park⁵ in 1901 of a paper entitled "The great bacterial contamination of the milk of cities. Can it be lessened by the action of health authorities?" contained information regarding the way in which bacteria entered and grew in milk. This report served as the basis of a very practical circular of information intended to teach dairymen simple principles of milk sanitation. Probably the first work done on a state-wide basis was that organized by H. W. Conn in Connecticut in 1908.

By the time the Laboratory Section of the American Public Health Association undertook to prepare the first Standard Methods Report on Bacteriological Methods for the Examination of Milk quite a series of cities had become interested in this problem, and Boston had

even established a count of 500,000 per ml. as the maximum permitted. The names of the seven men comprising the first committee appointed in 1905 to standardize methods recall some of the early centers of interest. These were S. C. Prescott (Boston), W. H. Park (New York City), F. H. Slack (Boston), H. L. Russell (Madison and Milwaukee), C. E. Marshall (East Lansing), F. C. Harrison (Montreal), E. C. Levy (Richmond). By the time the report was drawn up and adopted in 1910, 21 cities of the United States and Canada were represented by one or more individuals who had contributed enough to the success of the report to secure mention in the acknowledgments given.

By 1910, when the first Report of the Committee on Standard Methods for the Examination of Milk was printed, almost all of these and a few other cities were carrying out routine analyses of samples of milk as delivered. A few dealers in the Boston area and elsewhere had started routine monthly, or occasionally more frequent, analyses. Perhaps all told these cities and dealers were examining 50,000 samples of milk annually. These examinations consisted of agar plate counts and, especially in the Baltimore, Philadelphia, and Boston areas, of the microscopic examination of sediments obtained by centrifuging.

In 1925, a questionnaire was sent to as many laboratories as could be found that were doing routine milk control work. Two hundred and forty-one laboratories replied to the questionnaire, and the number of persons employed in milk work in these laboratories was stated to be 581. The number of samples examined yearly in public laboratories to determine the bacterial content of the milk by the agar plate method was more than 200,000, while laboratories maintained by the industry examined more than 1,000,000 samples.

Statistics were again gathered in 1929-30 in even more complete form from 342 public health and 125 commercial laboratories for the Committee on Standard Methods. Incomplete returns showed that in this year public health laboratories of the United States analyzed more than 540,000 samples of milk by the agar plate technic, with 28,000 in Canada, a total of 568,000. More than 294,000 samples were analyzed by the direct microscopic method in the United States and 2,500 in Canada, a total of 296,500. Using the methylene blue reduction method, 146,000 samples were analyzed in the United States and 5,000 in Canada, a total of 151,000. During the same

period, the laboratories maintained by the dairy industry in the United States analyzed more than 2,074,000 samples by the agar plate technic, while the industry in Canada analyzed 30,000 samples, a total of 2,104,000 samples. In the United States more than 750,000 samples were analyzed by the direct count technic, and 1,400 in Canada, a total of 751,400. The number of samples analyzed by the methylene blue reduction method was 538,000 in the United States, and 46,000 in Canada, a total of 584,000.

This rapid development of the use of laboratory methods in controlling the quality of milk shows no sign of abatement. Even with all of this work only a small part of the fluid milk supplies of the United States and Canada receives more than a cursory laboratory examination during each year.

Six editions of this report were issued under the title *Standard Methods for the Examination of Milk*. The scope of the work was broadened with the seventh edition to cover methods for the examination of other dairy products and the title was changed at the same time to *Standard Methods for the Examination of Dairy Products*.

Much comparative analytical work was done by early committees and these studies culminated in the publication of the first edition of the report in 1910. The committee at the time the report was published consisted of F. H. Slack, Chairman, W. H. Park, B. H. Stone, H. L. Russell, C. E. Marshall, and F. C. Harrison. In the years that followed immediately after this there was a demand that the agar plate technic be more definitely standardized by the adoption of a single time and temperature for incubation and the adoption of the more readily prepared and less expensive meat extract agar in place of meat infusion agar. The adoption of the meat extract agar and incubation at 37° C. for 48 hours was brought about at the 1915 (Jacksonville) meeting of the American Public Health Association. These changes were incorporated in the brief provisional report published in the *American Journal of Public Health*, 6:1315-1326, 1916, by a committee composed of M. P. Ravenel, Chairman, John F. Anderson, H. W. Conn, W. H. Park, and R. S. Breed, with B. H. Stone and W. R. Stokes as adjunct members. This committee worked in coöperation with committees of the Society of American Bacteriologists and of the American Dairy Science Association.

The third edition was prepared in 1921 by a committee consisting of W. H. Park, Chairman, M. P. Ravenel, R. S. Breed, John F. Anderson, and H. A. Harding, with B. H. Stone and W. R. Stokes as adjunct members. In an effort to make the report truly representative of American laboratory workers, this committee sought and obtained the additional coöperation of the International Association of Dairy and Milk Inspectors and others interested in the sanitary control of milk.

The fourth edition, published in 1923, was made a joint report on chemical and bacteriological methods in which the Chemical Methods for Milk Analysis compiled by the Association of Official Agricultural Chemists and the Standard Methods for Bacteriological Examination of Milk approved by the Laboratory Section of the American Public Health Association and other coöperating associations were combined in one volume. The bacteriological methods were prepared under the supervision of the Committee on Standard Methods composed of R. G. Perkins, Chairman, W. H. Park, A. B. Wadsworth, F. P. Gorham, H. W. Clark, N. MacL. Harris, E. O. Jordan, A. P. Hitchens, and G. W. McCoy, with R. S. Breed acting as Referee. The chemical methods were prepared under the supervision of the Committee on Editing Methods of Analysis of the Association of Official Agricultural Chemists composed of R. E. Doolittle, Chairman, B. B. Ross, A. J. Patten, J. W. Sale, G. W. Hoover, W. H. MacIntire, and of the Board of Editors of the *Journal* of that Association, R. W. Balcom, Chairman, R. E. Doolittle, W. W. Randall, R. B. Deemer, and W. F. Hand.

Through the courtesy of Ch. Porcher, Editor of *Le Lait*, the fourth edition was translated into French and published in *Le Lait*.

The fifth edition was published in 1929 through the coöperation of the associations previously mentioned. The Committee on Standard Methods of the American Public Health Association consisted of E. O. Jordan, Chairman, W. H. Frost, F. P. Gorham, N. MacL. Harris, G. W. McCoy, W. H. Park, R. G. Perkins, L. M. Wachter, and A. B. Wadsworth, with J. F. Norton as Secretary and R. S. Breed as Referee.

The preparation of the chemical methods was carried out under the supervision of R. E. Doolittle and R. W. Balcom of the Committee on Editing Methods of Analysis of the Association of Official

Agricultural Chemists, and of Julius Hortvet, the General Referee on Dairy Products of that Association.

The most active groups involved in the preparation of the sixth edition were the American Public Health Association and the Association of Official Agricultural Chemists. The work of preparing the manuscript for the bacteriological methods was carried out by R. S. Breed, Referee, with Mac H. McCrady, G. J. Hucker, and F. W. Fabian acting as Associate Referees under the supervision of a committee composed of A. W. Wadsworth, Chairman, N. MacL. Harris, Secretary, R. G. Perkins, J. F. Norton, G. W. McCoy, L. M. Wachter, W. D. Stovall, A. L. MacNabb, L. C. Havens, T. F. Sellers, D. T. Fraser, W. H. Park, and F. Lee Mickle. The manuscript for the chemical section was prepared by a special committee of the Association of Official Agricultural Chemists composed of E. M. Bailey, G. G. Frary, and F. C. Blanck.

Several important changes and additions in methods were made in the sixth edition which was printed in 1934. Directions for the Frost little plate and the Stewart-Slack procedures were omitted. Tentative methods were introduced for detecting coliform bacteria, hemolytic streptococci, thermophilic bacteria, and for making counts of bacteria in plain ice cream.

Because of a desire to develop reports in additional fields, all Standard Methods Reports developed by the Laboratory Section were, in 1934, placed under the supervision of a Coördinating Committee,⁶ with the following membership: A. Parker Hitchens, Chairman, J. F. Norton, R. S. Breed, W. D. Stovall, and F. Lee Mickle. The special committee that developed the seventh edition of the Dairy Products Report consisted of R. S. Breed, Chairman, Mac H. McCrady, A. H. Robertson, F. C. Blanck, C. A. Perry, R. V. Stone, A. J. Slack, and C. A. Abele, assisted by a group of Associate Referees as follows: F. W. Fabian, E. H. Parfitt, W. D. Tiedeman, G. J. Hucker, H. R. Thornton, C. S. Mudge, C. C. Carson, C. N. Stark, A. T. Thompson, I. F. Huddleson, and W. A. Hagan. Before the seventh edition of the Dairy Products Report was issued in 1939, R. A. Kelser and G. D. Cummings had been added to the Coördinating Committee. Bioassay Methods for Vitamin D Milk, and Phosphatase Methods for the Determination of Pasteurization, as well as Chemical Methods for Analyzing Dairy Products, were included in this edition. The Bioassay Section was

prepared by a committee of the Food and Nutrition Section composed of H. T. Scott, Chairman, F. D. Baird, J. W. M. Bunker, W. E. Krauss, E. M. Nelson, and W. C. Russell. The appendix outlining the technic for four different procedures for detecting phosphatase in milk and cream was developed by J. H. Shrader, while the Chemical Methods Section was prepared by a Special Committee of the Association of Official Agricultural Chemists composed of E. M. Bailey, Chairman, G. G. Frary, and F. C. Blanck.

A new formula for the standard agar used in milk and cream work was introduced with this edition, together with more definite specifications for the control of incubation temperatures. In addition, methods were given for detecting tubercle bacilli and undulant fever organisms in milk and cream. Included also were methods for the examination of butter. The inclusion of methods for the examination of ice cream and butter and the plan to develop the report so as to include methods for the examination of all frozen desserts including ingredients such as condensed, evaporated, and dry milks brought about a change in the title of the report from *Standard Methods of Milk Analysis* to *Standard Methods for the Examination of Dairy Products*.

The primary purpose of the publication of the eighth edition of this report in 1941 is to make it possible to include the methods for the examination of frozen desserts developed at the request of the Laboratory, and the Food and Nutrition Sections as expressed at the New Orleans meeting of the Association in 1939.⁷

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PART I. MICROBIOLOGICAL METHODS FOR THE EXAMINATION OF MILK, CREAM, AND BUTTER

LABORATORY, AND FOOD AND NUTRITION SECTIONS

American Public Health Association

A. SAMPLES AND SAMPLING EQUIPMENT

Authorized control officials shall take representative milk and cream samples at sufficiently frequent intervals to assure the consistent conformity of a supply to grade requirements. Because the quality of any given milk supply may vary from day to day, the maximum interval between sampling ought not to exceed 1 month. Take multiple samples to establish complete representation, if necessary. When taking samples, promptly identify each one legibly and indelibly with an official number or tag corresponding to the inspection record. Cool samples immediately and rush them to the laboratory for prompt examination as hereinafter required. If the interval between sampling and examination exceeds 4 hours, record on the analysis report the time of sampling and the time of examination.

To determine the conformity of new supplies to sanitary regulations and for grade assignments, examine, preferably by the agar plate method, not less than four samples of milk or cream, each taken on a different day.

I. STERILIZATION

Sterilize sampling and plating equipment whenever possible with dry heat in a hot air sterilizer, at not less than 160° C. for not less than 1 hour. Determine temperatures accurately. Use slightly higher temperatures, or preferably longer periods, if the sterilizer is crowded with glassware or other equipment. Sterilize media, dilution waters, and materials that may be charred at dry air sterilization temperatures, such as rubber, cork, cotton, paper, etc., at 121° C. (15 lbs. steam pressure) for not less than 20 minutes,

in an autoclave equipped with an accurate thermometer and an accurate pressure gauge. To insure sterility, rely upon the temperature in the autoclave rather than upon the pressure. Use a longer sterilization period if the materials are packed closely or if attempts are made to sterilize large quantities of material at one time. Observe this precaution especially if the materials have a relatively low temperature when put in the autoclave, thereby causing a delay in the prompt heating of the mass. Permit all air to escape from the steam sterilizing chamber before raising the pressure. Loosen all stoppers to permit the passage of steam into dry, stoppered containers when autoclaved. In emergencies, suitable pressure cookers may be substituted for autoclaves.

Where there is any chance of careless operation of sterilizers, or pressure cookers, keep a record of the time when the oven or autoclave reaches a sterilizing temperature, the minimum temperature used, and the time when the source of heat is discontinued for each lot of materials sterilized.

II. RETAIL SAMPLES

For retail samples of milk or cream or other similar dairy products in containers take samples from or of a representative percentage of each size of unopened container as delivered to the consumer. Take not less than 1 pint if the sediment test is to be made on the same sample. Protect caps and lips of sample containers at all times from contamination, with tight-fitting, waterproof coverings—especially when bottles of milk or cream are shipped to a laboratory.

III. WHOLESALE SAMPLES

For producers' and dealers' wholesale samples (usually in 5 or 10 gallon cans) immediately after delivery to the purchasing agency, transfer not less than 10 ml. of the well mixed milk or cream from the previously unopened container, preferably with a suitable sterile metal or glass tube to a sterile sample container (clean screw-cap vials, glass-stoppered bottles or other leak-proof containers), allowing the complete contents of the tube to drain into the latter. Use a similar procedure for removal of weigh-vat samples and for samples to determine the efficiency of pasteurization. For convenience of packing for shipment, use preferably sample bottles sizes 0.5 to 1.0 oz. or optional sizes 2.0 to 8.0 oz. Remove each sample with

a separate sterile sampling instrument and reserve the entire contents for the sample. Avoid filling the container more than two-thirds full.

Metal (usually aluminum*) tubes, approximate size, outside diameter 0.8 cm., wall 0.1 cm., length at least 61 cm. (24 in.) made of straight-sided seamless tubing, are recommended. If aluminum tubes are used, it is better to sterilize them in the autoclave because at dry air sterilization temperatures they are apt to become soft and flexible. A 5 per cent trisodium phosphate solution in steaming hot water is satisfactory for cleaning aluminum tubes, but avoid the corrosive effect of prolonged contact between alkali and metal. Rinse tubes thoroughly to remove all traces of cleaning solutions.

IV. STIRRING BEFORE SAMPLING

Immediately before removal of the sample, agitate the milk or cream thoroughly and vigorously with a sterile stirrer long enough to reach the bottom of the container or, if practical, by repeated forceful inversions of the vessel. Do not take official samples from containers of unstirred milk or cream or from the stream as the unstirred liquid is poured from the can. Any stirrer already in the can may be used for agitation. Do not use the metal or glass sampling tube as an agitator unless no other stirring instrument is available. Always use sampling tubes, never dippers, where there is any question about the thorough agitation of the milk or cream. If a sampling tube is used as a stirring instrument, remove it completely from the liquid before taking the sample and insert it, not too rapidly, to the bottom of the container, with the top of the tube open. Place the finger securely over the open end, withdraw the tube and its contents and aseptically transfer the entire liquid to a sterile sample bottle or vial. Wash and sterilize each tube and sample container before re-use.

Samples may be removed from individual cans by dipping a 0.5 or a 1.0 oz. sterile container in the well mixed milk, or caught in a similar sterile container as the milk is poured into the weigh-vat, providing satisfactory facilities are available for stirring the milk or cream immediately before emptying the can. Use long-handled clamps to hold the sterile containers. Subject the clamp to conditions of practical sterilization between successive samples. Handle

* Obtained from the Aluminum Company of America, Pittsburgh or New Kensington, Pa. (Ask for aluminum tubing made from 2 SH or 53 ST alloy); The Whitehead Metal Products Co., 303 W. 10th Street, New York, N. Y.; The Summerill Tubing Co., Bridgeport, Montgomery Co., Pa.; and from various laboratory and supply houses.

the sterilized sample containers and caps aseptically also. Satisfactory facilities for stirring cans of milk or cream consist in providing one can connected with a continuous flowing source of clean cold water and (1) another can containing hot water kept continuously at not less than 180° F. by passing live steam through it, or (2) another can containing a hypochlorite solution (strength maintained at not less than 100 parts per million of available chlorine) for the practical sterilization of the stirrer. Rinse the stirrer first in cold water and then allow it to stand in the hot water or chlorine solution while making preparations to take the next sample. (Figure I.) Where plate counts are made on the collected samples, plate samples of the sterilizing solution each day when sampling is completed.



FIGURE I—Sampling milk in cans with a sterile aluminum thief. Samples placed in screw-capped vials and placed in a round tin container. Tin container placed in shipping case with cracked ice. In the background two milk cans may be seen, one filled with hot water (containing steam hose and stirrer), and the other with cold water for use in collecting samples.

V. WEIGH-VAT SAMPLES

Do not take samples to determine conformity to public health regulations from the mixed milk of several cans after the milk has

been dumped into the weigh-vat, because correctionary and punitive measures to be legal must be based on samples taken from previously unopened containers.

In sampling practice at milk plants where premiums are paid for low count milk, it has been found that individual cans selected at random from among several delivered by a dairyman frequently are not representative of an entire delivery. Even when samples are examined as often as semi-weekly, the results are still open to a question of fairness. Sampling from all the cans delivered is impracticable because of the excessive number of samples to be taken in so brief a time at milk receiving stations. Because of more complete representation, the taking of weigh-vat samples is regarded as practical and satisfactory for the purpose of determining premiums to be paid dairymen for the production of low count milk, providing frequent examinations are made. Contamination from deliveries of milk previously dumped into the weigh-vat has been shown to be so slight, if any, and so infrequently positively demonstrable, that weigh-vat sampling is preferable to sampling a single can of either night or morning milk, or even both, from a delivery consisting of more than one can.¹

When weigh-vat sampling is practised, and the bacterial counts on the samples are used as a basis for premium payments to producers, pour the milk or cream into the weigh-vat in such a manner as to mix it thoroughly and take the sample immediately. Further requirements are (1) maintenance of a correct list of the patrons in the order of their deliveries, (2) as complete drainage of the weigh-vat as possible—total residual milk to be less than one pound—and (3) use of strainers or other equipment on weigh-vats in such a manner that they do not interfere with the thorough mixing of the milk in the vat or contribute undue contamination thereto.²

VI. COOLING THE SAMPLES

Cool all samples as promptly as possible to a temperature between 32° and 40° F., and hold within this temperature range until analyzed. The results of the analysis of any sample the temperature of which has exceeded 45° F. during a storage period of 4 hours may be unreliable. Use trays or other suitable receptacles provided with compartments or baffles to hold the sample containers in a vertical position.

Do not insert a thermometer into a sample before the bacterio-

logical sample is removed. If necessary to determine the temperature of a sample before removal of the portion for bacterial analysis, insert the thermometer in a liquid held in a similar container which has been treated identically as the sample to be examined for its bacterial content. Temperatures of samples may be determined from the milk immediately after the removal of the portion for analysis; but necessary handling prior to its removal may increase the temperature of the milk slightly, especially if the original sample is relatively small—less than 4 oz. Where conditions under which samples are received vary from day to day, it is desirable to test each lot of samples and record the observed temperature on the analysis report.

VII. SHIPMENT AND TRANSPORTATION OF SAMPLES

When shipping and transporting unopened bottles of retail milk and cream, use large well constructed boxes which will permit the use of sufficient refrigerant to maintain the desired temperatures until the containers arrive at their destination. For the shipment of wholesale samples or of those to determine the efficiency of pasteurization, use preferably screw-cap vials or bottles, sizes 0.5 to 1.0 oz., with tops molded or ground smoothly and caps with proper skirt length to secure a leak-proof contact between the cap and the glass. Parcel post or express shipments of samples in ground-glass-stoppered bottles, with tops protected by tight fitting metal, metal foil, viscose or parchment paper caps, are apt to be unsatisfactory. Directly transport samples in such containers to the laboratory, preferably under the personal supervision of the laboratory worker. Avoid rise of liquid refrigerant above the shoulders of such bottles if the liquid is in direct contact with the container, and always keep the bottle in a vertical position during storage and transportation.

Samples collected from individual quarters of the udder or if collected at different stages of the pasteurization process and carried directly to the laboratory, may be collected in test tubes which are tightly closed with sterile rubber or cork stoppers. Do not use cotton plugs to close sample containers.

VIII. SHIPPING CASES

Provide proper trays, boxes, or other rigid equipment designed for the adequate and prompt cooling, protection, and transportation of samples at all times (Figure I). When quart or pint bottles of milk or cream are to be shipped in water containing cracked ice, securely protect the entire top of the bottles with waterproof paper or metal foil. When screw-cap vials or glass-stoppered bottles are used, place them in one-tier-deep, metal, water-tight containers for further protection during transportation. Cover these water-tight containers completely with cracked ice in well constructed wood, metal-lined wood, or metal boxes. Other types of cases with adequate insulation for the shipment of samples cooled to near their freezing point are useful, providing the entire case is likewise cooled before shipment is made. Prevent samples from freezing when dry ice is used as a refrigerant. To insure proper handling during transportation, attach handles to cases to encourage shipment in an upright position and label cases conspicuously THIS SIDE UP on the appropriate surface.

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B. AGAR PLATE METHOD

The agar plate method for estimating bacterial populations consists of counting the isolated visible bacterial growths (colonies) which develop in and on the surface of a specially prepared, semi-transparent, jelly-like nutrient medium after a suitable dilution of the milk or cream has been mixed with a small amount of the liquefied sterile medium immediately prior to the latter's solidification. The colonies are counted after holding the solidified mixture for 48 hours optionally at 37° C., or at 32° C., and the count so obtained is reported in accordance with provisions herein for a "Standard Plate Count."

I. APPLICATIONS AND LIMITATIONS

The agar plate method is commonly used for the examination of milk and cream as delivered to the consumer and of samples to determine the efficiency of pasteurization. If the presence of bacteria that fail to grow on the plates is suspected, check the accuracy of the plate count either with the direct microscopic method or by incubation of additional plates at suitable temperatures as described later (Section B, VI, 2, p. 30).

Examine raw milk samples as received at milk stations normally by either the direct microscopic method (described in Section C) or by the methylene blue reduction test (described in Section D). Use the agar plate method in all cases where premiums are paid to dairymen for milk with standard plate counts (Section A, V, p. 11) less than 50,000 per ml. The payment of premiums based on bacterial counts for different grades of milk requires a specially high degree of accuracy in the analytical work to insure justice to the producers.

The agar plate method has been used in an official way longer than other methods for estimating microbic populations and can be used on all types of dairy products including both milk and cream. On pasteurized products, supplement its use by the direct microscopic method for the more complete identification of types of bacteria that find the standard agar and incubation temperature unsuitable for growth. The agar plate method, as compared with other

recognized procedures, is especially adapted to the examination of samples containing very few bacteria and also for the plating of samples at successive stages of processing to detect slight contaminations.

1. SOURCES OF ERROR

Agar plate "counts" per ml. are "estimates" of numbers rather than exact counts, because of certain inherent limitations and because only a portion of a ml. is used in preparing plates. As such they are, like all estimates, subject to certain well known and recognized errors whose size can be controlled in part by the number of duplicate samples analyzed.¹ Among these errors are (1) failure of some of the bacteria to grow because the incubation temperature, reaction, or composition of the medium is not suitable, (2) inaccuracies in the measurement of the quantities used, (3) mistakes in counting, recording observations, computing results, etc., and (4) incomplete sterilization or contamination of glassware and dilution waters. In the interests of reproducibility of results on the same sample, it is essential that all laboratories follow the standard procedure exactly.

Investigations^{2, 3} clearly demonstrate that these largely controllable errors are less apt to cause serious misconceptions of the accuracy of the results than those errors which are due to the uneven distribution of bacterial populations in the original substance to be examined. Bacteria in milk and cream usually occur in groups of from two to several hundred individuals. The customary shaking of samples and dilutions is an inadequate method of breaking apart the clumps so that, under optimum conditions, the count of the colonies on the plate represents the number of isolated individuals and groups of two or more bacteria that exist in the final dilution water that are capable of growing under the conditions used. Thus the colony counts from the plates are always smaller than the total number of viable cells present. This error would not be troublesome if the groups were of constant average size; but observations indicate that the groups in ordinary market milk vary in size so that they contain an average of from 2 to 6 cells, while unusual samples, *i.e.*, those containing long chain streptococci, may have groups with an average of 25 or even more individual bacteria. The irregularity of this error, whose size is in no way indicated by the appearance of the plates, should be kept in mind when interpreting the results obtained.

II. APPARATUS AND MATERIALS

1. PIPETTES

Use straight-sided bacteriological pipettes conforming to the following specifications:



FIGURE II

- a. Pipette delivering 1.1 ml.
 b. Pipette delivering 2.2 ml.
 c. Pipette delivering 11 ml.

Standard A.P.H.A. 1.0 ml. pipette: total length of pipette not less than 275 mm.; length graduation to tip 125 to 175 mm.; length graduation to upper end at least 100 mm.; length of tip 10 to 15 mm.; outside diameter of tubing 6.5 to 7.5 mm.; bore of tubing 2.7 to 3.2 mm.; bore at tip 1.75 to 2.0 mm., wall at tip 1.0 to 1.3 mm.; delivered contents, last drop of undiluted milk blown out or last drop of diluted milk touched out, 1.0 ml.; tolerance ± 0.025 ml. To allow for the adhesion of milk and milk dilutions on the glass under the specific technic hereinafter described (Section B, IV, 4, p. 26) such pipettes should be constructed to contain 1.075 ml. of water at 20° C.⁴ Studies indicate that the use of pipettes which do not conform to the above specifications, and especially the lack of uniform manipulation of the same, will cause variable results.

If a pipette which will deliver both 0.1 ml. and 1.0 ml. is desired, use a pipette with a mark placed above the 1.0 ml. graduation so that the volume delivered as determined by the difference between the two graduations shall be 0.1 ml. (Figure IIa.)

When making a 1 to 200 dilution, use the standard 1.0 ml. pipette with a graduation mark below the

1.0 ml. mark at the 0.5 ml. point so as to permit the successive delivery of two 0.5 ml. quantities, each in a 2 second interval. Pipettes shall be graduated to contain 1.18 ml., 1.075 ml., and 0.54 ml. of water at 20° C. to deliver 0.1 ml., 0.5 ml., and 0.5 ml. quantities successively. These graduations shall be marked successively downward 1.1 ml., 1.0 ml., and 0.5 ml. with a pigment which sharply contrasts with the color of the milk or its dilutions.

Two suggested modifications are: 1. A pipette graduated for two successive 0.5 ml. deliveries may be made with two successive 0.1 ml. graduations above the 1.0 ml. mark for duplicate 1:200 and 1:1,000 dilution transfers directly to the plates in a continuous operation without refilling the pipette. 2. A pipette to deliver two 0.1 ml. and two 1.0 ml. volumes (total capacity 2.2 ml.) for use when preparing four plates, two of each of two different dilutions.⁵

Pipettes that deliver 1.0 ml. and 0.1 ml. quantities from the same pipette are convenient for preparing plates with 1:100 and 1:1,000 dilutions from the 1:100 dilution water blanks. Likewise 1:10,000, and 1:100,000 or similar combinations can be prepared from the proper dilution water blanks with a single pipette.

An 11.0 ml. pipette, hereinafter called the standard 11.0 ml. bacteriological pipette, modified to deliver 11.0 ml. in 3 to 4 seconds, is suited for making a 1 to 10 dilution in a 99 ml. water blank when examining either a very low count milk or viscous substances like cream. The 11.0 ml. pipette may be graduated to contain 11.6 ml. of water at 20° C.

Any laboratory using pipettes not conforming to standard A.P.H.A. specifications shall be responsible for their accurate calibration and manner of use when measuring the milk and its dilutions according to standard technic.

Recalibration of bacteriological transfer pipettes used in laboratories making counts is required in New York State and is desirable under all conditions. The error of calibration on the standard 1.0 ml. pipette and its modifications shall not exceed a tolerance of ± 0.025 ml. A minus error in count approaching 10 per cent is produced by the use of pipettes graduated *to contain* rather than *to deliver* 1.0 ml.

2. DILUTION BOTTLES

For the 99 ml. quantities of dilution water, use dilution bottles

with a capacity of about 150 ml., made preferably of resistant glass and marked indelibly at the 99 ml. graduation level. If other quantities of dilution waters are used in the bottles, indelibly mark the graduation levels desired on containers of correspondingly appropriate capacities. If desired, 9.0 ml. dilution blanks may be prepared in 1 oz. bottles, test tubes, or other containers. Suitable bottles for the 99 ml. quantities of dilution waters are shown in Figures III-V.

Figure III illustrates a 6 oz. bottle of flint glass fitted with a special rubber stopper that is not readily blown from the bottle during autoclaving.

Figure IV shows a ground-glass-stoppered bottle which is sterilized empty in a hot air oven and filled later with sterile water measured with a sterile graduate as needed from a supply of freshly



FIGURES III-V—Dilution Bottles with Non-Absorbent Stoppers.

sterilized water. Bottles of this and other similar types are sometimes filled by drawing sterile water into them under aseptic conditions from hospital-size sterilizers. In some laboratories the supply is sterilized in 1,000 ml. Erlenmeyer flasks. If bulk sterilized water is employed, use the dilution blanks within a few hours after preparation.

Figure V shows a square 6 oz. bottle made of properly annealed resistant glass. The Pyrex bottle is very resistant to breakage while the flint glass bottle is intermediate in resistance between Pyrex and ordinary glass. Both of the better types of bottles are much more resistant to the solvent action of dilution waters than the cheaper ordinary glass bottle.

Bottles of this type may be closed with single-hole rubber stoppers into which 3 in. glass rods of the proper diameter are fixed. Where the end of the rod is mushroomed and allowed to project about 1 in. above the stopper it permits convenient handling of the stopper by means of the little finger. Similar glass rods are sometimes inserted in solid rubber stoppers in an eccentric position by making a hole with a heated ice pick and quickly inserting the glass rod in the hole. The purpose of these special devices is to allow the stoppers to be inserted loosely in the necks of the bottles and prevent their popping out of the bottles as a result of pressure from within during autoclaving. Ordinary solid rubber stoppers may be used, provided a rigid sheet of material is placed immediately over them or is suspended a slight distance above them. Bottles with screw cap closures may be used where leak proof closures are assured (these may require new liners in caps after each use). Cotton plugs for closing dilution water blanks are unsatisfactory.⁶

3. PETRI DISHES

Use Petri dishes, frequently called plates, 100 mm. in diameter and 15 mm. deep. Provide dishes with flat bottoms which are free from bubbles, scratches, and other defects. Prevent breakage and dust contamination following sterilization by storing and handling glassware, especially plates, in suitable containers. Remove plates from containers when these are placed in the incubator chamber in order to permit plates to reach the incubation temperature promptly.

4. HOT AIR OVENS

Use hot air sterilizing ovens, with shelves properly spaced, of suitable size to prevent overcrowding of the interior, of proper construction to obtain satisfactory uniform temperatures in the oven, and provided with suitable vents for the prompt acquisition of sterilizing temperatures and for insertion of a thermometer to determine the temperature within. After determining the variations of temperature within the oven, use a thermometer at all times to assure the complete sterilization of equipment.

5. AUTOCLAVES

For sterilizing materials in moist heat, use autoclaves of suitable size to prevent overcrowding of the interior and of proper construction to obtain uniform and adequate temperatures during steriliza-

tion. Equip autoclaves with accurate thermometers, accurate pressure gauges, and properly adjusted safety valves, and provide proper temperatures either by direct connection to a saturated steam line or by gas or electrically heated steam generators.

6. THERMOMETERS

Use thermometers of proven accuracy only. Check the accuracy of the less expensive laboratory thermometers by comparing their scale readings at several temperatures with the corresponding temperature readings on a thermometer which conforms to the specifications of the National Bureau of Standards. Instruments for controlling and recording temperatures automatically are satisfactory providing proper temperatures are established and maintained.

7. INCUBATORS AND INCUBATOR ROOMS

At present two types of incubators are regarded as satisfactory—the water-jacketed type, and the anhydric type with low temperature electric heating units properly located and insulated in the walls and floor of the chamber.⁷ Keep the wall space filled with water if a water-jacketed type is used. Incubators equipped with high temperature heating units, frequently characterized by the absence of sufficient resistance, are regarded as unsatisfactory since such sources of heat cause localized over-heating and excessive drying of the agar, with a consequent failure to form colonies. Incubators so heated may be made to operate satisfactorily in many cases by replacing the high temperature units with wiring arranged to operate at a low temperature.

Provide shelves at suitable distances from each other and the sides of the chamber to assure uniformity of temperatures. A proper arrangement of shelves, heating units, or a series of baffles often reduces the range of temperature variations within the incubator. Determine the temperature variations in the incubators by placing accurate thermometers at several points on the shelves with the incubator filled with poured milk plates to its maximum capacity consistent with good incubation practice. Do not use anhydric incubators with inside dimensions less than 20 in. wide, 20 in. deep and 24 in. high (or equivalent space) because smaller incubators are apt to be crowded more frequently, causing temperature irregularities. An incubator of the above size should incubate from 160 to 200 plates

satisfactorily, with an inch space both between adjacent piles and between the walls and the piles.

Incubator rooms are satisfactory, provided areas can be found in the room where the temperature variation is within the specified tolerance limits. When constant temperature rooms are used for milk plates, record the daily range in temperature in the portion of the room where the plates are incubated.

Keep laboratory type incubators in rooms where temperatures do not vary excessively (50° to 80° F. preferred). Special rooms are desirable for incubators, the temperatures within which can be kept a few degrees below the desired incubation temperature. Where the room temperature is so high as to interfere with the desired incubation temperature, provide facilities for cooling the room or the incubator chamber.

8. WATER

Prepare all culture media and reagents with distilled water. Use tap water preferably, or buffered phosphate dilution water prepared in conformity with Section K, page 126 for making dilutions of milk and cream.⁸ Do not use waters which are known either to have an abnormally high mineral content or to be toxic. Test tap and distilled dilution waters occasionally to determine their freedom from chlorine, copper, and other substances which may be toxic to bacteria. Determine the suitability of dilution waters by preparing plates from a series of samples immediately after the dilutions are made and periodically thereafter from the same dilution waters at intervals up to 30 minutes. Do not use dilution waters which cause a definite trend toward lower counts within the 30 minute interval

9. BEEF EXTRACT

Use Bacto beef extract, or other beef extracts giving equivalent results. Do not substitute meat infusion for beef extract.

10. PEPTONE

Use Bacto-tryptone, or other peptones giving equivalent results.⁹

11. AGAR

Use granulated or shred agar of the best quality. Agar may contain obligate thermophilic bacteria that are not killed by ordinary

steam sterilization.¹⁰ The presence of thermophiles may be detected by incubating the prepared media at 45° to 55° C. and noting whether bacterial colonies develop on it.

12. SUGARS AND OTHER CHEMICALS

Use fermentable carbohydrates and other analytical reagents of the highest purity.

13. STANDARD NUTRIENT BROTH

Prepare standard nutrient broth by adding 5 gm. of Bacto-tryptone and 3 gm. of beef extract to 1,000 ml. of distilled water. Add 5 gm. of glucose for all ordinary purposes. Other sugars may be substituted or added for special purposes.

14. STANDARDS FOR HYDROGEN-ION CONCENTRATION

Use accurate and reliable standards to determine the hydrogen-ion concentration colorimetrically or determine the reaction of the media electrometrically using dependable equipment.

15. MEDIA-MAKING EQUIPMENT

Dissolve the ingredients and prepare the media in glass, or other suitable utensils which will not contaminate the liquids with toxic materials, such as copper, zinc, antimony, chromium, etc. Wash containers promptly and thoroughly to remove remaining particles of agar. Dried particles of agar are difficult to redissolve and may resemble bacterial colonies when incorporated with the next batch of agar which is poured into the plates.

III. STANDARD NUTRIENT AGAR

1. COMPOSITION AND PREPARATION

Use standard tryptone-glucose-extract-milk agar of the following composition for routine work.⁹ (See opposite page.)

Use either granulated or shred agar. After weighing, if desired, soak the shred agar in distilled water to remove salts and dirt. Drain the agar before use.

Prepare nutrient agar by adding 3 gm. of beef extract, 5 gm. of tryptone, 1 gm. of glucose, and 15 gm. of agar (undried market product as purchased) to 1,000 ml. of distilled water. Dissolve the

COMPOSITION

Agar, best quality, not oven-dried.....	1.5 per cent
Beef extract	0.3 per cent
Tryptone	0.5 per cent
Glucose	0.1 per cent
Distilled water	

Reaction pH 6.6 to 7.0

Preferred reaction..... pH 7.0

1 per cent skim milk is to be added just before final sterilization in all cases where dilutions greater than 1:10 are to be made.

agar by boiling over a free flame and stirring to prevent burning on the bottom of the container, or by exposing the mixture of ingredients in a flask or other suitable container to the action of flowing steam in a steam chest or in the autoclave. Make up lost weight with distilled water. Where the dilutions that are to be made are greater than 1:10, add 10 ml. of good quality skim milk just before final sterilization. The milk may be kept in stock by storing in sterile condition in test tubes, bottles, or flasks.

An equivalent amount of spray process skim milk powder may be substituted for the skim milk. Dissolve 10 gm. milk powder in 100 ml. water. Use 10 ml. of this reconstituted milk per liter of agar. Care should be taken where powder is used to avoid troublesome precipitates.

Bacto-tryptone-glucose-extract agar, dehydrated, or other dehydrated agars of the composition specified above may be used.¹¹ Add the skim milk, if required, during the preparation of the agar.

2. ADJUSTMENT OF REACTION

State the reaction of culture media in terms of the hydrogen-ion concentration as expressed in pH values. Determine the hydrogen-ion concentration either electrometrically or colorimetrically.¹²⁻³⁰ Colorimetric methods are usually satisfactory for this purpose.

Test the reaction of the medium as described below and adjust same if it falls outside the pH range of 6.6 to 7.0; preferred reaction pH 7.0. To test the reaction, add 5 ml. of distilled water at 50° F. to each of two clean test tubes, similar in size, shape, and color to the tubes used for color standards—6 × 0.5 in. tubes recommended. Withdraw 10 ml. of medium to be adjusted and add 5 ml.

to each of these tubes. To one of these add 5 drops of a solution of an indicator which will adequately cover the desired range—brom thymol blue recommended. The amount of indicator in the test solution should be the same as in the standard. Using a comparator block superimpose the tube containing the diluted medium plus the indicator over the tube of distilled water and superimpose the tube of diluted medium without the indicator over the color standard of the desired pH. Titrate the tube of diluted medium plus the indicator with an accurate 1 to 10 dilution (1 part of NaOH solution to 9 parts of H_2O) of an approximately normal NaOH solution until the color viewed through the distilled water tube matches the color of the pH standard as observed through the diluted medium without the indicator. Calculate the amount of the normal NaOH solution which must be added to the medium to reach the desired pH. Add the amount so calculated to the agar, mix thoroughly and again test the reaction. If the reaction is incorrect, make the proper addition of acid or alkali to correct for the error. If the errors are excessive, it may be advisable to discard the particular batch of agar.

3. STERILIZATION AND STORAGE

Before final sterilization, bring the medium to a boiling temperature, stirring frequently, restore lost weight with hot distilled water and clarify if deemed advisable. The necessity for clarification depends upon the intended use, the inherent cloudiness and the preference of the individual worker. Clarify using any method which yields a medium suitable for the detection of all bacterial colonies and which at the same time will not remove or add nutritive ingredients, such as by centrifugalization, by sedimentation, or by filtration of the melted agar through paper, cotton, cheese cloth, or towels. Do not clarify with egg albumen.

After clarification distribute the medium in suitable containers and sterilize it in the autoclave (Section A, I, p. 7). Flasks and bottles are convenient receptacles for the sterilized agar although some prefer to pour the melted agar from test tubes containing the measured 10 to 12 ml. quantities. Limit the amount of agar in large containers so that no part of the agar shall be more than 2.5 cm. from the glass, except that containers more than 5.0 cm. in diameter may be filled to a depth not exceeding 5.0 cm. If flasks or bottles are stored, prevent excessive evaporation and con-

tamination during storage by securely fitting, pliable, metal foil, parchment paper, or viscose caps over the cotton plugs and necks of the containers. If crown cork and seal type caps are used, such hazards are reduced to some degree. To prevent recontamination, sterilize the coverings chemically before placing them over the necks of the sterilized containers, or fit them over the caps before sterilization.

Remove a portion of each batch of sterilized agar aseptically immediately before use and determine the final reaction thereof. Record this as the official reaction of the medium. The hydrogen-ion concentration during sterilization is apt to increase resulting in a pH decrease of from 0.2 to 0.4. After solution of the ingredients, the minimum exposure to heat which is consistent with freedom from precipitates and with absolute sterility is essential. Check the sterility of each lot of agar whenever a portion thereof is used by pouring at least one control plate at the end of the plating process. Check the sterility of the dilution water by pouring a control plate with each series of samples examined and for each lot of dilution blanks used with these samples.

IV. DILUTIONS

1. UNIFORMITY OF DILUTION WATER MEASUREMENTS

Fill the dilution bottles with the amount of water necessary to produce 99 ml. or other desired amounts after sterilization. If desired, 9.0 ml. blanks may be used to avoid the transfer of 0.1 ml. amounts. Predetermine experimentally for the particular autoclave and the method of operation the exact amount of water to add to the bottle before autoclaving so that the amount remaining will be as desired.^{31, 32} After sterilization and before use observe each bottle to determine the deviation from the required amount. Discard any bottle with a variation exceeding ± 2 ml. from the 99 ml. quantity. If smaller quantities of dilution waters are used in the bottles, the tolerance error should be proportionately smaller. Automatic measuring devices for dilution water are satisfactory if the same tolerance is maintained. Use dilution blanks promptly especially if they are prepared from measured quantities of bulk sterilized water added aseptically to sterile bottles.

2. MARKING PLATES WITH DILUTIONS USED

Before making dilutions, arrange the plates in order and identify them with the sample number and the dilution to be used. Plates may be marked with a number corresponding to the number of ciphers in the dilution used, as follows: using sample No. 16 as an illustration—16-0 for no dilution of the milk, 16-1 for a 1:10* dilution, 16-2 for a 1:100 dilution, 16-3 for a 1:1,000 dilution, etc.

3. AGITATION OF SAMPLE

Agitate the contents of each bottle thoroughly and vigorously immediately before the removal of any portion of any sample or its subsequent dilution. Preferably agitate the contents of a full container by pouring the contents back and forth repeatedly under aseptic conditions between the original container and another container previously sterilized in the laboratory for the occasion. If practical, agitate wholesale and process samples by shaking in containers which are not more than two-thirds full. When the milk is in the vials or bottles, or in the dilution bottles, shake each container rapidly 25 times, each shake being an up-and-down excursion of about a foot, time interval not exceeding 7 seconds. Before opening the sample container, remove all material from the closure which may contaminate the milk or cream. If deemed advisable, wipe the top of the container with a sterile cloth which is saturated with alcohol.

4. MEASURING THE SAMPLE

Do not wipe or drag the pipette across the lip and the neck of the vials and dilution bottles when the original 1.0 ml. of milk and its subsequent dilutions are removed. Make transfers carefully because each drop of milk or of the dilution water falling into a bottle or a plate from the outside of the pipette may be expected to increase the count of the successive dilution water or transfer by at least 5 per cent.

When measuring the milk, let the column drain from the gradua-

* In this expression of dilution and similar ones used throughout the text, the first figure means one part of milk and the second one the total volume after the addition of the milk to the dilution water. The correct form is 1 part of milk in 99 parts of water. The inaccurate expression is commonly used because the count found on the plate made from the dilution water is to be multiplied by 100.

tion mark to the apparent rest point of the liquid in the tip of the pipette and then blow out the last drop as quickly as possible.⁴ Ordinarily the drainage and complete deposition requires between 2 and 3 seconds.

For cream samples, preferably weigh 1.0 gm. aseptically into a sterile butter-boat or directly into the dilution bottle on an accurate cream test torsion balance or one of equivalent sensitivity. (The use of butter-boats is described in detail in Part II, 1, B, II, 1, p. 150.) If a butter-boat is used, transfer it and its contents to the dilution bottle. Owing to differences in the specific gravity of cream depending upon varying compositions and viscosities at different temperatures, measurement of cream with a pipette is never entirely satisfactory. If a pipette is used, withdraw a sufficient quantity to assure the delivery of 1.0 ml. of cream, well mixed but free from air bubbles, preferably at 20° C. (68° F.). Before depositing in the dilution bottle, let the column drain to the apparent rest point of the liquid in the tip of the pipette, and then blow out the last drop as quickly as possible. Predetermine for each laboratory and for each manner of use, the quantity of cream which will be required if a 1.0 ml. pipette is used to deliver 1.0 ml. of cream. Dilution blanks, if used at a temperature from 30° to 32° C., aid in the rapid dispersion of the cream in the dilution water. If the initial dilution is made at this temperature, prepare the plates promptly.

When measuring milk and cream dilutions, hold the end of the pipette at an angle of 45° against the Petri dish or against the neck of the dilution bottle or the rod in the dilution bottle. Allow the diluted milk or cream to drain from the graduation mark to the apparent rest point in the tip of the pipette, and then touch once against a dry spot on the glass.⁴ Ordinarily this operation requires between 2 and 3 seconds. Use care to raise the Petri dish cover only just enough to insert the pipette.

Use a separate sterile pipette in making transfers from each sample and from each dilution. Do not rinse pipettes in dilution waters.

5. SELECTING PROPER DILUTIONS

Select dilutions so that the number of colonies on at least one plate will be between 30 and 300.^{33, 34} In instances where the analysis is to determine whether the count exceeds the limits 10,000 to 300,000 per ml., prepare at least two plates per sample. Use at least two different dilutions, preferably 1:100 and 1:1,000, although

1:50 or 1:200 may be prepared where conditions make these dilutions desirable. Odd dilutions are apt to lead to errors of computation more frequently than dilutions made in direct multiples of tens. If the standard plate count is expected to be under 10,000 per ml., prepare plates from 1:10 and 1:100 dilutions.

In exceptional cases where previous records indicate that a supply is consistently uniform so that a single plate with between 30 and 300 colonies is obtained, the custom of making a single plate may be used. Rigidly restrict this application to the frequent examinations of relatively low count milk and cream supplies which have been examined over a sufficient time to establish their uniformity beyond a reasonable doubt. In case of failure to conform to the specified range of colonies per plate, procure additional samples and prepare suitable dilutions when plating.

6. CLEANING PIPETTES

Because improperly cleaned pipettes may cause incomplete deliveries, thoroughly wash pipettes with suitable detergents after each use and periodically soak them overnight in a strong cleaning solution. A bichromate-sulfuric acid cleaning solution may be prepared by adding 50 gm. sodium bichromate to 200 ml. of water in a glass or earthen container, and then cautiously add 300 ml. of sulfuric acid, commercial grade or Babcock testing grade. Wash acid-treated glassware thoroughly in alkaline waters and then thoroughly and repeatedly rinse in clean water. Tests of the rinsed glassware for presence of residual acid or alkali, may be made by using appropriate indicators, such as brom thymol blue, Andrade's, or other suitable indicators.

V. PLATING

Melt the prepared nutrient agar in boiling water, flowing steam, or under steam pressure. The melted agar usually may be kept in a fluid condition for a short time in a constant temperature water-bath or incubator at 45° C. without causing a precipitate or a decrease in the pH in the agar. Use a separate flask of water into which a thermometer extends as a temperature control of the medium in the water-bath or incubator. Do not depend upon the sense of touch as an index of the proper temperature for pouring the agar.

After depositing the desired portions of the milk and cream in the Petri dishes, introduce from 10 to 12 ml. of the liquefied agar at 41° to 44° C. into each plate within an interval not exceeding

20 minutes after the first transfer was made from the sample. Lift the cover of the dish just enough to insert the pipette and to pour the agar. Sterilize the lips of all media containers before the first pouring and periodically thereafter by direct exposure to a flame. Mix the agar and the sample thoroughly and spread it evenly over the bottom of the dish by rotating and tilting the dish carefully without splashing the mixture over the edge of the plate. After distribution, solidify the agar quickly on a level surface by cooling (if necessary), invert the plates and place them in the incubator³⁵ at once. Where clay tops are used, inversion of plates during incubation is unnecessary.

If the interval between sampling and plating exceeds 4 hours, record on the analysis report the time the sample was taken and the time of plating.

VI. INCUBATION

1. STANDARD PROCEDURE

Arrange plates or piles thereof on the shelves of incubation chambers so that they are separated horizontally from each other and from the top and walls of the chamber by at least 1 inch. The number of plates permissible in a pile and consistent with uniform temperature distribution is dependent upon the type of incubator used. Always place piles directly over each other on successive shelves, *i.e.*, do not stagger piles.

Two temperatures of incubation are recognized as standard:

1. Incubation for 48 hours at 37° C.
2. Incubation for 48 hours at 32° C.

At 37° C., slight increases in temperatures materially lessen the number of visible colonies that will develop unless organisms of the types that grow at high temperatures are present. When using 32° C., it is also important to prevent fluctuations in incubation temperatures, although slight variations in temperature have less effect on the magnitude of the count.⁷ The temperature of incubation used shall be determined by the enforcement officials having jurisdiction.

Because a temperature reading at the top of the incubator chamber is invariably an incorrect index of the temperature throughout the chamber, determine the temperatures by placing not less than two thermometers, one on the top and one on the bottom shelf, or

additional thermometers at other intermediate points. Record the observed temperatures daily on the top and bottom shelves during those periods when the incubator is in use for milk plates. Take the temperatures from water or other liquids contained in small vials or flasks at the desired positions in the incubator as air temperatures are unreliable. Prevent the evaporation of the liquid by securely fitting a cork or stopper around the thermometer stem and into the neck of the vial or flask. Accurate maximum and minimum recording thermometers or automatic temperature controlling and recording devices may be found desirable and may be used.

Avoid excessive humidities in the incubator, a probable cause of spreaders on the plates; and prevent excessive ventilation and air circulation, a cause of excessive drying of the agar. The agar should not lose more than 15 per cent of the weight within 48 hours. Plates kept in incubators that are operating properly will show no apparent signs of dryness in 4 days.

2. DETECTION OF BACTERIA THAT DO NOT GROW AT 32° OR 37° C.

Milk, cream, and other dairy products may contain large numbers of bacteria that do not find the temperatures used for routine incubation favorable for growth. Some bacteria find temperatures between 32° and 37° C. too hot for growth, others find these temperatures too cold for growth. Therefore, standard plate counts even when 32° instead of 37° C. incubation is used may at times fail to reveal more than a fraction of the total bacterial population.

The easiest way to determine whether large numbers of bacteria are being overlooked is to compare the numbers found by the standard plate method with the numbers found in microscopic preparations made as specified later. However, it is necessary to incubate agar plates at lower or higher temperatures than those used in the routine work if it is desired to detect the number of low or high temperature organisms present in any given sample.

Low temperature organisms frequently occur in cream, ice cream mix, or similar products that have been held in cold storage for several days or for longer periods. To find these bacteria, incubate additional plates for 3 to 5 days at 18° to 25° C. If true psychrophilic bacteria only are sought, incubation should be at temperatures as low as 5° to 10° C. for a sufficient length of time to permit visible colonies to develop (10 to 14 days or even longer).

High temperature organisms frequently occur in pasteurized products. They are especially apt to be found in the deposits on filter cloths through which hot milk has passed, in foam on tanks of hot milk or cream, in deposits on the walls of utensils in which milk is heated, and in milk and cream samples taken at the end of pasteurization runs. Incubate additional plates at 45° C. for 48 hours if the presence of organisms whose optimum temperature of growth is above 37° C. is suspected. If true thermophilic bacteria only are sought, incubation should be at 55° C. for 48 hours. The maintenance of proper moisture conditions in incubation chambers operated at high temperatures is important.

VII. COUNTING PLATES

Count all colonies on plates that have developed at the end of 48 hours incubation, tolerance ± 3 hours. If impossible to count within the time limits, place the plates in the refrigerator at a temperature less than 10° C. (50° F.) for a period not exceeding 16 hours. Do not make this a routine practice.

1. SELECTING SUITABLE PLATES

After incubation of two plates per sample, one from each of two dilutions, it is expected that the number of colonies on at least one, and perhaps occasionally both plates, will be between 30 and 300.^{33, 34} The optional use of more than two plates per sample is allowed. These extra plates may be either of the same or of different dilutions. Count all colonies including those of pin point size and include them in the officially reported count.

Report as the standard plate count the number of colonies on the plate yielding between 30 and 300 colonies (or the average if the number on both plates is within the limits) except (1) where more than one plate per dilution is made and (2) where there are no plates with colonies within the 30 and 300 limit.

(1) If two or more plates are made from the same dilution and only one has colonies within the 30 to 300 limit, use the others of the same dilution to determine the average count. In certain rare instances, this might include the counts from successive dilutions as some plates of the lower dilution might have less than 300 colonies and others more than 300, while some plates from the higher dilution might have more than 30 colonies and others less. In such cases use the average of all of the plates.

(2) Use the plate with nearest 300 colonies for the standard plate

count if the dilutions selected are too low to give colonies within the 30 to 300 limits. Use counts from plates with less than 30 colonies only when no other plates are available.

Regardless of any of the above requirements, if the number of colonies developing on the suitable plates from successive dilutions is such that the higher count is more than twice the lower count, use the count or counts from the plate or plates which give the lower count. Normally these will be the plate or plates with the number of colonies nearest 300.

If the number of colonies on the plates to be counted exceeds 300 per plate, count a representative portion of the plate and estimate therefrom the total number. Such plates are admittedly overcrowded and the counts are less than they should be. If the number of colonies per plate is not within the prescribed limits, *i.e.*, 30 to 300 per plate, the results are less dependable. If none of the plates show colonies, report the result of the analysis as unsatisfactory.

2. COUNTING AIDS

Count the colonies under uniform and properly controlled illumination and normally with the aid of magnification. A reading glass 4 to



FIGURE VI—Quebec Colony Counter

5 inches in diameter magnifying about $1\frac{1}{2}$ diameters is the simplest and most essential aid. Supplement its use by a non-glaring, ruled guide plate, rulings in squares preferred, placed beneath the Petri dish. Examine carefully any doubtful specks in the agar using additional magnification where required to distinguish colonies from dirt specks. Take measures to prevent the presence of specks of undissolved agar, undissolved milk powder or precipitates from the water used in the dilution bottles.

An approved counting aid, known as the Quebec Colony Counter,³⁶ (Figure VI) is recommended, the advantage of which is that both reflected and transmitted artificial light is directed on the colonies in the Petri dish under controlled conditions. Small colonies stand out in striking contrast to the agar. In order to insure uniformity of counting conditions, illumination equivalent to that provided by the Quebec Colony Counter shall be employed.

Use a hand tally (Figure VII) for recording mechanically the number of colonies counted. If an automatic colony dotting counter (Figure VIII) is used, proper and constant illumination shall be employed.

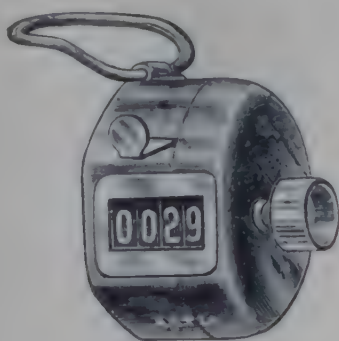


FIGURE VII—A convenient type of hand tally that is frequently used in counting colonies on plates.

3. PERSONAL ERRORS IN COUNTING

Technicians who cannot duplicate their own counts on the same plate within 5 per cent and those of other technicians within 10 per cent should discover the cause and correct such disagreements. The three chief causes of inaccurate counting are carelessness, impaired vision, and failure to know what should be counted.



FIGURE VIII—An automatic counter. Colonies may be automatically recorded and marked in ink by using this counter.*

4. SPREADING COLONIES

At least three distinct types of spreading colonies, each of which may have a single original source, are recognized. The first type is a chain or chains of colonies which appear to arise from the disintegration of more or less compact clumps of bacteria found in the milk. If but a single chain of colonies is formed count the chain as one. If

* Obtainable through Dr. M. O. Robinson, Polytechnic Institute, Auburn, Alabama.

several chains are formed, count the approximate number of chains. Do not count each individual colony as one.

In the second and third types of spreaders, count each spreader as a single source. Discard plates where the spreader covers more than half of the plate. The second type is the spreader that develops in the film of water between the agar and the bottom of the dish, and the third is the one that forms in the film of water at the edge or over the surface of the agar. The two latter types are true spreaders and develop because of accumulation of moisture at the point from which the spreader originates. Identical types of bacteria do not develop spreading colonies when the surface of the agar is relatively dry. Do not count plates that have developed spreaders if there is any indication that the spreader has repressed the formation of other colonies. Any laboratory with 5 per cent or more of the plates $\frac{1}{4}$ covered with spreaders should take immediate steps to prevent their formation.

5. ESTIMATING COLONIES ON CROWDED PLATES

Where the number of colonies on the plate appreciably exceeds 300, count colonies in portions of the plate representative of colony distribution and estimate therefrom the total number on the plate. Where there are from 5 to 10 colonies per sq. cm. area, count the colonies in from 12 to 14 different areas, selecting, if representative, the 6 consecutive squares diagonally across the plate and the 6 consecutive squares, at right angles to the first diagonal. Where there are more than 10 colonies per sq. cm. area, count colonies in 4 such sq. cm. areas, selecting, if representative of colony distribution, the 4 areas near the center. Multiply the average per sq. cm. area as found by the appropriate factor, to obtain the number of colonies per plate. Use Petri dishes with flat bottoms only to avoid an error in selecting representative squares at the center of the plate for estimation of the count.

6. ERRORS CAUSED BY DIFFERENCES IN PLATE DIAMETERS

Since errors are introduced by the variations in the inside diameter of the bottom of the Petri dishes, determine what factor should be used when multiplying the average number of colonies per sq. cm. to obtain the number per plate when a fraction of a plate is counted. Because 91 mm. has been determined by actual measurement to be the average inside diameter of the bottom of the recommended type of Petri dish, normally multiply the average number of colonies

per sq. cm. by 65. If, however, the average inside diameter is only 90 mm., multiply by 63.5; if 92 mm., multiply by 66.5.³⁷

If the Petri dish is placed over a counting plate ruled with radiating sectors, the colonies in opposite sectors may be counted. This procedure eliminates the influence of differences in the diameters of the dishes and usually equalizes differences in the thickness of the layer of agar due to irregularities in the bottoms of the dishes.

VIII. REPORTING COUNTS

Because agar plate counts do not represent the total number of bacteria present, do not report them as showing the "number of bacteria per ml." Accurately speaking, counts from agar plates give the estimated number of colonies that would have developed per ml. of milk on the agar under the conditions specified if an entire ml. of milk had been examined. Because this complete statement is cumbersome, and also because in each case an unknown ratio exists between the colony count and the total number of bacteria, the practice of speaking of plate counts as showing the number of bacteria per ml. is incorrect and should be avoided. This confusing statement, however, is no longer so frequently seen now that the direct microscopic method permits an estimated count of the individual bacteria.

Report all plate counts obtained by the standard technic in the form "Standard Plate Count 20,000 per ml." rather than as "20,000 bacteria per ml." The expression "Standard Plate Count" is an abbreviated form for saying "an estimated count of 20,000 colonies per ml. as obtained by standard methods." Always record whether 32° or 37° C. incubation has been used.

Data showing the actual percentage of error in these counts have been difficult to obtain, and have only been obtained by means of comparisons made between microscopic and agar plate counts.^{3, 38, 39} Since colonies on the agar plates develop either from isolated bacteria or from groups thereof, and since the average number of bacteria in groups which may form colonies varies from a little more than one individual to 25 or more individuals per group,^{40, 41} there is no assurance that agar plate counts of the same magnitude really represent even approximately the same number of individual cells. Even if the average number of individual bacteria per group is as low as 1.5, the very next sample of milk with the same standard plate

count may be from a milk in which the average number of individual bacteria per group is 3.0 or even 6.0 or more, so that the second sample may actually contain twice, four times, or even more bacteria per ml. If two samples are compared, the one having the higher count probably does contain the larger number of bacteria, and this probability can be made a practical certainty by the examination of a series of samples. Therefore, it is required that a series of samples, at least four (Section A, p. 7) be examined before judgment shall be rendered as to the general quality of a given milk supply.

Do not publish counts from individual samples of milk or cream. If publicity is given analytical results, either as standard plate counts or as scores, give equal ratings to all dealers or persons selling milk or cream that conform to the standards fixed by regulations, *i.e.*, where a regulation calls for a count of less than 10,000 per ml., report all counts from supplies with less than this figure as less than 10,000 per ml., etc.

Keep a record of the dilutions used and the number of colonies developed on each plate that is counted but render the reports in round numbers only, *i.e.*, in case there are 35 colonies on the 1:100 plate, report the count as "Standard Plate Count 3,500 per ml."; in case there are 252 colonies on the 1:100 plate, report it as "Standard Plate Count 25,000 per ml." If two or more counts are averaged, do not give a fictitious idea of the accuracy of the standard plate count by using more significant figures than are found in the numbers averaged, lowering the count where the figure to be dropped is 1, 2, 3, 4, and raising it where the figure to be dropped is 5, 6, 7, 8, or 9. If plates developing less than 30 colonies must be used, report the count merely as "less than 300" if the 1:10 dilution has been used, "less than 3,000" if the 1:100 dilution has been used, etc. Those wishing to be still more conservative will use a form of report such as "Standard Plate Count between 10,000 and 30,000 per ml.," etc.

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C. DIRECT MICROSCOPIC METHOD

The direct microscopic method¹ consists of an examination with the aid of a compound microscope, of stained films of milk and cream dried on glass slides. Five applications some of which are not too clearly defined may be mentioned: (1) Rapid examination of films for the purpose of assigning the samples to certain grades; (2) the making of actual numerical estimates (a) of individual bacteria or (b) of clumps of bacteria counting each isolated bacterium and groups of two or more as clumps; (3) rapid detection of poor quality milk at milk receiving stations and recognizing by means of bacterial types the most probable cause of high counts in raw milk; (4) empirical derivation of an estimated plate count from a clump microscopic count; and (5) examination of pasteurized milk to determine whether bacteria are present that do not develop colonies on agar plates.

I. APPLICATIONS AND LIMITATIONS

The microscopic method offers the most rapid routine technic for obtaining a general opinion of the bacterial condition of the sample. The rapidity with which the information can be obtained on an individual sample in each of the fields of application—usually within 15 minutes—and particularly the ability to identify different bacterial types and groupings in the milk, make this method the most valuable for correcting unsanitary conditions by directing the attention promptly to the most probable cause. The presence of stained bacteria and other cellular elements as revealed under the microscope is a convincing proof of their existence, and frequently suggests the underlying cause or causes of bacterial condition of the samples. While sanitary codes generally recognize limiting bacterial counts for milk and cream in terms of standard agar plate counts, bacterial counts made by the microscopic method can be used equally as well for this purpose, at least for raw milk. When such use is made of microscopic counts, the counts so obtained are to be considered on their own merits, no attempt being made to interpret the microscopic count in terms of the agar plate count.

1. ASSIGNMENT TO GRADES

The direct microscopic technic is used chiefly in routine control

work for making rapid estimates of the number of bacteria present rather than for making time consuming counts of the number of individual bacteria present. Where large numbers of bacteria are present and are uniformly distributed on the film, the examination of a single field will indicate the general character of the sample. More microscopic fields must be examined where none or only a few bacteria are present, as uneven distribution of bacteria in clumps may deceive the person making the examination into thinking that the milk is better or worse than it really is. For the purpose of grading, the microscopic technic is just as satisfactory for the examination of high quality (low count) milk as for poor quality milk because it is as easy to recognize the absence as it is to determine the presence of bacteria. Smears prepared from low count milk contain so few bacteria that sometimes none can be found even after a tedious search, while smears made from a high count milk show numerous bacteria in each field examined. Normally it is possible to determine whether the milk or cream is in excellent, good, unsatisfactory, or very unsatisfactory condition without counting, and hence no counts are required except in so far as they are needed in borderline cases to establish the correct assignment to a grade.

In the examination of samples of pasteurized milk or cream, the fact that these products have been heated sufficiently to kill and thereby destroy many but not all of the bacteria must be kept in mind. Moreover, thermophilic or even thermoduric bacteria may have increased in number during the heating of these products. Contamination from equipment after pasteurization may also have added its quota of living bacteria. If pasteurized milk or cream has been stored at low temperatures, it must also be remembered that low temperature (psychrophilic) bacteria may have grown in these stored products. Since there can be any combination of the above possibilities, cautious interpretation of observations is essential.

2. ESTIMATES OF THE NUMBER OF BACTERIA

The actual counting of bacteria and the grading of milk samples by the microscopic method are two distinct uses of the technic which often have been confused. No other method of examination permits counting individual bacteria. Because of this advantage, the technic may be used in research work where estimates of the

total number of bacterial cells may be needed. Actual counting of the bacteria involves more painstaking efforts than are required for grading. In samples containing relatively few bacteria, the search of a large number of fields required in the interest of accuracy fatigues the technician because of the close observation required and reduces the number of samples which can be examined in a limited time.

Some workers prefer to estimate the number of clumps of bacteria present instead of the number of individual bacteria, and when estimates of clumps in borderline cases are made carefully, such procedure produces satisfactory results. Actual counts or estimates ordinarily are required in milk control work only in so far as it is essential to assure conformity to limiting bacterial count standards or to inform as to the degree of variation from such standards.

When making estimates of the bacteria in samples of pasteurized milk and cream, observe the precautions outlined in the preceding application with reference to pasteurized samples.

3. RAPID DETECTION OF POOR QUALITY MILK AND RECOGNITION OF CAUSES OF HIGH COUNTS

This application is closely allied to that involving the examination of films for the purpose of assignment of samples to certain grades. The method is most valuable and used most frequently for the rapid detection of poor quality milk at receiving stations. No counts are required and the examination of even the first field indicates the general quality of the milk.

The presence of specific bacterial types in the stained films indicates with reasonable accuracy whether high counts in raw milk are caused by infected (usually by streptococci) udders, poorly cleaned and inadequately sterilized utensils, or growth subsequent to milking (faulty cooling); *provided* not more than 10 gallons of milk are mixed as in an individual can, and that the age and temperature of the milk are known. Where larger amounts of milk are mixed, interpretations of the past history of the milk are made with less assurance of accuracy.

4. INTERPRETATION OF MICROSCOPIC COUNTS IN TERMS OF ESTIMATED PLATE COUNTS

Because the agar plate method and the direct microscopic method

for obtaining bacterial counts yield such characteristically unlike results, it has not been possible to devise an accurate system for interpreting one count in terms of the other. The methods of counting are so different that assigned values for the interpretation of results in terms of the other method yield results quite different from those actually obtained by analysis. Official counts and grades by the microscopic method are, therefore, to be considered on their own merits and should not normally be interpreted in terms of standard plate counts. Counts made with the microscope may be of the number of individual bacteria, or of isolated bacteria and clumps (unseparated groups) of cells. The former count correlates better with observed sanitary conditions and the interpretations usually ascribed thereto. The latter agrees better with the standard plate count as this is dependent on a count of colonies which may have arisen either from a single isolated bacterium or from a clump.

Comparisons show that there is no constant ratio between the total number of individual bacteria present and the plate count. Hence, even the most frequent ratio⁴ between the standard plate count and the individual count (1:4) is not recognized as an accurate basis for the interpretation of one count in terms of another, since the majority of the ratios vary widely from the most frequent ratio.

If an estimated plate count is derived empirically from microscopic preparations, it should be made on the basis of a count of separate cells and clumps of bacteria, allowance being made for the probable breaking up of such clumps in the dilution waters used in the plating procedure. Clump counts made in this manner and standard plate counts should agree reasonably well where the bacteria present are of a type that exists in milk as single individual cells, and are capable of growth on standard agar at the incubation temperature used.⁵ Such a group or clump count can, with experience, be made to correspond fairly well with the plate count. Bacteria killed by heat lose staining ability so quickly that they cause little difficulty⁶; even if recognizable, dead bacteria are just as significant as, and sometimes more significant than, living bacteria when judging the past history of milk. Accurate counting of clumps involves the individual judgment to such an extent that skill can only be acquired with practice and by continual comparison of the group count with the standard plate count.

5. EXAMINATION OF PREPARATIONS OF PASTEURIZED MILK

The method is also valuable for detecting the type of contamination and growth in pasteurized products; and as a ready means of determining the presence or the absence of types of bacteria that fail to grow on plates made by standard methods. There are two important groups of bacteria that fail to grow on the present standard agar when the plates are incubated at 32° or 37° C.; and these bacteria may be and frequently are present in excessive numbers although the standard plate count gives no indication of their presence. The bacteria in question are the thermophilic, usually large rod types that find 32° or 37° C. too cold for growth; and cold temperature (psychrophilic), frequently fluorescent types of bacteria that find 32° or 37° C. too hot for growth. Both are readily detected under the microscope where well stained bacteria should be regarded as having probably been alive at the time the preparation was made.

6. COMMON SOURCES OF ERRORS IN COUNTS

Routine microscopic counts, like all bacterial counts, are to be regarded as estimates of numbers only. They cannot be made with absolute accuracy, even with the most careful technic. Errors may arise from inaccuracies in measurement of the minute quantities of milk examined at any one time, from faulty staining or preparation of the slides, from mistakes in observation, and the like. These limitations, while important, are not difficult to overcome in sufficient measure to make microscopic grading a satisfactory method for controlling the quality of milk. Since actual counts of bacteria themselves can be made only in this way, obviously carefully made estimates of individual bacteria as seen under the microscope give the truest picture of the total number of bacteria in milk that can be obtained by any technic.

II. APPARATUS AND MATERIALS 7

1. SAMPLES AND SAMPLING EQUIPMENT

Submit retail samples for analysis in the original unopened containers. Take wholesale



FIGURE IX
Capillary
pipette
delivering
0.01 ml.

samples and those to determine the efficiency of pasteurization as directed (Section A, III, p. 8). Provide proper mixing, cooling and transportation facilities for the samples (Section A, IV, V, VI, VII, and VIII, pp. 9-13).

Icing of samples may be omitted where chemical preservatives are used; for example, a drop of a 40 per cent formaldehyde solution to each 10 ml. of milk in the sample bottle. Add the preservative either to the milk as the sample is taken or to the empty containers a few hours before use. Store containers in a cool dark place if the preservative is put in the empty bottle or vial. If preservatives are used, do not allow a compact cream layer to form before preparing the films. Containers for chemically preserved samples shall be clean and preferably sterilized before use. Containers used for samples which are not chemically preserved shall always be sterilized before use.

2. PIPETTES

Use accurately calibrated capillary pipettes of the type hereinafter described and illustrated in Figure IX, for measuring the 0.01



FIGURE X—Calibrated loop for use in measuring milk

ml. quantities of milk and cream.^{7, 10} The most satisfactory form of 0.01 ml. pipette for use in preparing slides for microscopic examination is made from a straight piece of thick walled capillary tubing with a bore of such a size that the single graduation mark is from 40 to 60 mm. from the tip. Pipettes made from capillary tubing with a background of dark blue glass for the column of milk are satisfactory. The tip shall be blunt and of such a form that it will discharge milk cleanly without running back on the side of the tip. Calibrations shall be to deliver 0.01 ml. and not to contain 0.01 ml.

A pipette calibrated to contain 0.1395 gm. of mercury will discharge 0.01 ml. of milk at 20° C.

Do not use especially calibrated loops (Figure X) for measuring 0.01 ml. of milk and cream if counts or grades are made for official control purposes. They may, however, be used with satisfaction where the purpose of the work is educational in character.

3. GUIDE PLATE AND SLIDES

Use special glass or cardboard guides (a convenient size is 2 by 4½ inches, with 16 square or round 1 sq. cm. areas inscribed thereon, Figure XI) to indicate the area on the slide to be covered by each 0.01 ml. of milk or cream.

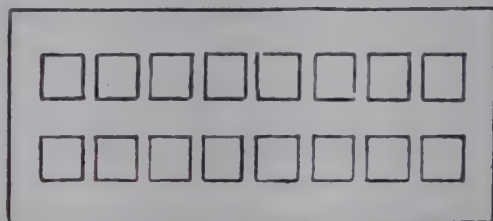


FIGURE XI—Special (2 by 4½ in.) Guide Plate

Spread the films evenly on glass microscopic slides (convenient sizes are 1 by 3, 2 by 3 or 2 by 4½ inches). Slides (Figure XII) should be provided with etched or ground margins to permit legible and indelible labeling.

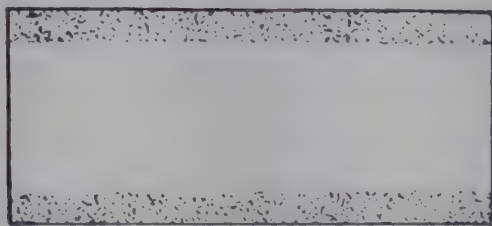


FIGURE XII—Special Microscope Slide (2 by 4½ in.) with Ground Glass Margins for Labeling

Thoroughly cleaned, discarded photographic negatives or other glass of similar quality provide a cheap source of slides when cut to the size desired, usually 2 by 4½ inches. An emery wheel may be used to grind a suitable margin for labeling. If desired for reference after the preparations are made and examined, file the slides as ordinary library cards are filed.

4. STAINS AND STAINING

Use stains certified by the Commission on the Standardization of Biological Stains to be satisfactory for the purpose intended. Use chemicals of the highest purity. Any convenient form of staining jar is satisfactory (Figure XIII).

If large numbers of slides are prepared, place the stain preferably in rectangular glass jars or trays and dip several slides simultaneously by placing them in a special holder provided with a handle (Figure XV).

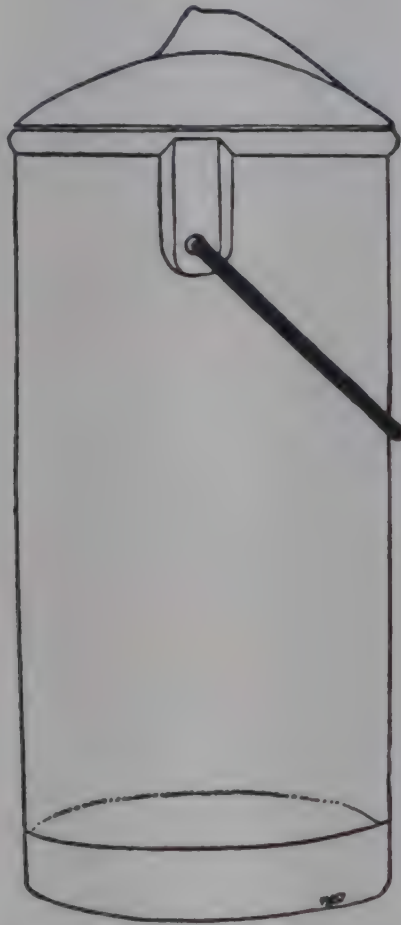


FIGURE XIII—One Type of
Wide Mouth Specimen Jar
Used in Staining Slides

5. MICROSCOPE AND ACCESSORIES

Use a compound microscope equipped with a 1.9 mm. ($1/12$ inch) oil immersion objective, a substage actuated by a rack and pinion and carrying an Abbe condenser with iris diaphragm, and an ocular (or paired oculars for use with a binocular microscope) that permits adjustment of the draw tube to a field of the desired diameter.



FIGURE XIV—Samples of milk (at left) from which preparations are to be made for direct microscopic examination. Guide plate and necessary equipment for making slides in center and at the right.

If it is at all possible, lenses that have not previously been adjusted by the manufacturer to yield a field of the desired diameter, should be returned to him for adjustment. This can be accomplished by a slight change in the size of the opening in the reducing diaphragms in the ocular or oculars. If lenses cannot be changed in this way, secure a field of the proper diameter by adjusting the length of the draw tube.

Both 6.4x and 10x oculars (or paired oculars for the binocular microscope) are useful. Use a special mechanical stage when the 2 by 4½ inch slides are used. This permits a wide range of slide movement. Determine the diameter of the field of the microscope with a stage micrometer ruled in 0.1 and 0.01 mm. divisions. Provide a standard microscope lamp, or some equivalent light source, as a constant source of artificial illumination. Use immersion oil between the slide and the objective.

Use a binocular microscope with paired oculars adjusted to give the desired field diameter when large numbers of samples are to be examined. Manufacturers of binocular type microscopes normally adjust tube lengths and paired oculars for use at the most common interpupillary distance and when requested standardize microscopes so that the field area is limited to one of the diameters specified below.

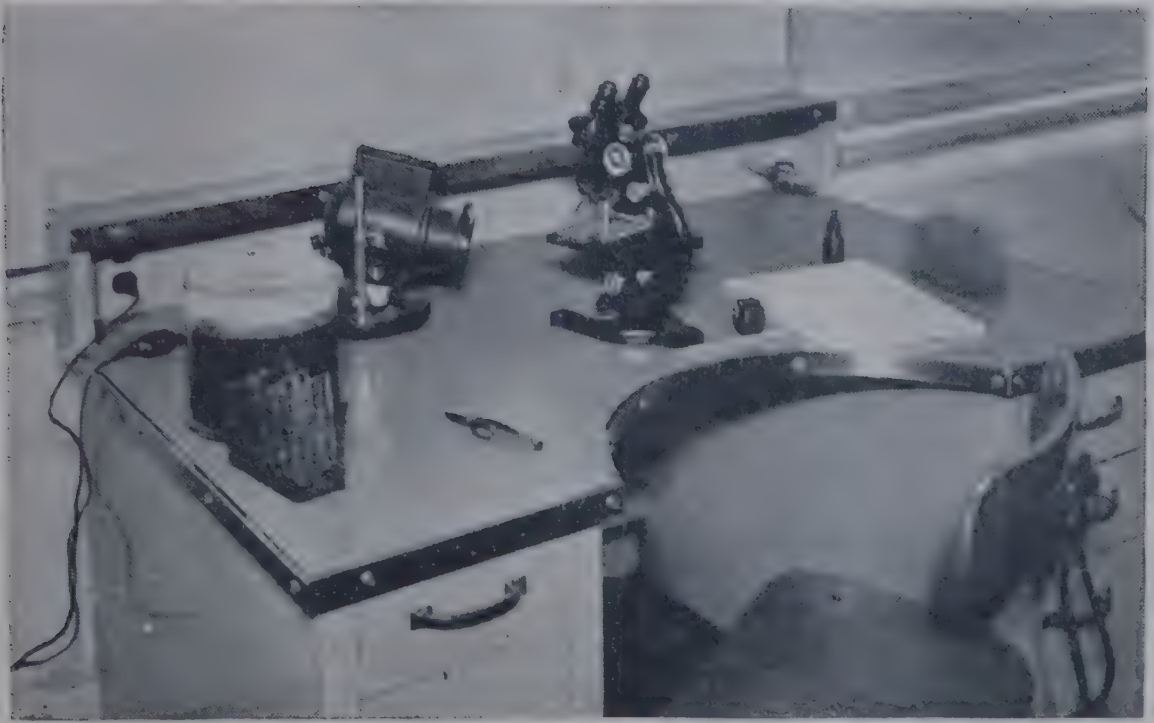


FIGURE XV—Basket containing prepared slides (at left) ready for staining by use of staining solution in adjacent round jar. Microscope with slide ready for counting the bacteria in center with record sheet and tally counter at right of microscope.

Measurements are made from the point of the most frequent interpupillary distance. Where counts are to be made by individuals with interpupillary distances varying widely from the average, paired ocular diaphragms should be prepared especially for their own use, or their counts should be corrected in terms of the microscopic field actually examined.

III. ADJUSTMENT OF MICROSCOPE

1. ILLUMINATION

When these adjustments cannot be made by the manufacturer of the microscope, adjust the microscope so that the tube length permits maximum optical resolution and fit a collar on the draw tube at the proper place, if necessary, to insure its use in this position. The sharpest definition of objects is obtained with American made microscopes when the tube length is 160 mm.—some European made microscopes 170 mm. Adjust the light source to secure maximum optical resolution.

2. FIELD AREAS

Use a special ocular diaphragm or an ocular micrometer disc (Fig-

ure XVI) to limit the field examined to the desired size when accurate counts are desired. The area of the field of the microscope determines the value which is to be used as a multiplier of the average number of bacteria per field to obtain the count per ml. of milk or cream. This multiplier is called the microscopic factor. The formula for calculating the microscopic factor is $M.F. = \frac{xy}{\pi r^2}$. Let $x = 100$ sq. mm. (area covered by the 0.01 ml. of milk), and $y = 100$ (the number of 0.01 ml. portions of milk in 1.0 ml.). If the diameter is 0.206 mm., the radius (r) is 0.103 mm. With this diameter, the microscopic factor is in round numbers 300,000. Field diameters measuring approximately 0.206 mm., 0.178 mm., 0.160 mm., and 0.146 mm. give microscopic factors of 300,000, 400,000 500,000, and 600,000 respectively.

A 500,000 factor can usually be obtained with a 10x ocular with the least deviation from a draw tube length giving maximum optical efficiency without the use of a special ocular diaphragm. Under similar conditions, a 6.4x ocular usually gives a 300,000 microscopic factor. By reducing the area examined with an ocular disc with a circle inscribed thereon (Figure XVI), a microscopic factor of

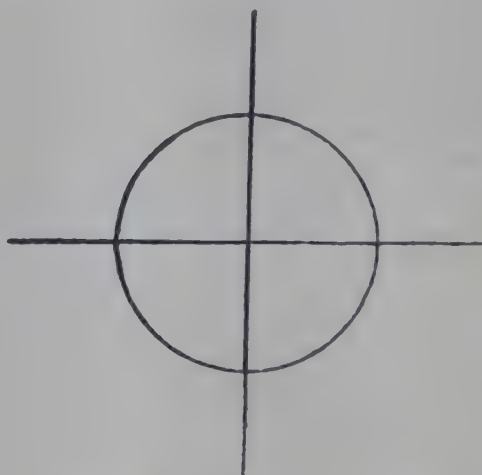


FIGURE XVI—Form of Guide
Lines Used on Special
Ocular Micrometer

600,000 can be obtained with a 6.4x ocular with the draw tube approaching maximum optical resolution.

When purchasing new microscopes, request the manufacturer to

provide a satisfactory standardization at one or more of these diameters. Microscopes purchased without standardization may be returned to the manufacturer with a request that they be standardized at one or more of the diameters specified to obtain maximum resolution. Check the accuracy of the manufacturer's field diameter adjustment with a micrometer slide ruled in 0.1 and 0.01 mm. If the adjustment is for a field measuring 0.206 mm., each field is an area of 1/3,000 sq. mm. (actually 1/3,000.37 sq. mm.) which permits the examination of the stained milk solids in essentially 1/300,000 part of a ml. of milk or cream on the stained preparation.

IV. PREPARING THE FILMS

1. USE AND CARE OF PIPETTES

Physical cleanliness of pipettes and slides is very important but absolute sterility is unnecessary. Use aseptic technic and keep the glassware free from foreign materials. Dip the slide in alcohol and flame it if sterility is desired. Use a single pipette and rinse thoroughly in fresh, warm, clean water (40 to 45° C. or 105 to 115° F.) between samples. After rinsing, wash out the small amount of water left in the bore in the subsequent milk sample. This procedure which may add a small number of bacteria to each sample introduces only a theoretical error, tests showing that the added bacteria cannot be detected subsequently. After use, fill the pipette with a cleaning solution and store in the same solution. The commonly used mixture of sulfuric acid, bichromate and water (Section B, IV, 6, p. 28) is satisfactory for this purpose.

2. MAKING THE FILMS

Place the slide over the guide plate. Before removing the sample, legibly and indelibly identify each space indicated by the guide plate with a number or some other symbol on the etched margin of the slide wherein the milk or cream will be deposited. This identification on the slide shall correspond to that assigned to the sample of milk or cream. After thorough agitation of the sample (Section B, IV, 3, p. 26), draw the milk or cream up into the pipette above the graduation mark. Use a clean towel to wipe the exterior of the pipette and to absorb the milk at the tip in order to reduce the column to the graduated portion. Completely deposit each 0.01 ml. of milk or cream in the proper place on the clean glass slide

with the capillary pipette. After each deposit, spread the liquid over an exact sq. cm. area with a clean bent needle, using the point of the needle as much as possible for spreading the film. Clean the needle by wiping with a clean cloth between samples. Work carefully but rapidly to prevent bacterial growth during the process of making the films.

After spreading the film, dry the preparation in a warm place upon a level surface protected from dust and insects. Prevent detectable bacterial growth on the slide by drying completely within 5 minutes. Do not heat too rapidly. Otherwise the preparations may crack and peel from the slide during later treatments.

If proper precautions are taken, films of heavy cream may be prepared that adhere to the glass as well as do milk films. These flatten during the alcohol treatment and stain as satisfactorily as do milk films. Such films cannot be prepared, however, unless precautions are taken (1) to dissolve all of the fat out of the film, and (2) to treat the film with alcohol and dry it in such a way as to flatten the film until it is thin and transparent before the slide is placed in the stain. It is difficult if not impossible to prepare satisfactory heavy cream films from single dip stains.

The best way to assure the complete removal of the fat from the film of cream is to give it a double treatment in xylene, drying the film after each treatment. Likewise, give the film a double treatment with alcohol, drying after each treatment. If drops of fat appear on the film during the alcohol treatment, it indicates that the film has not been completely defatted. In such cases the dried film should be returned to the xylene, for an additional treatment. Renew the xylene more frequently when extracting fat from cream than when extracting fat from milk films.

3. REMOVING THE FAT AND FIXING THE FILMS

After the first drying, dip the slide in xylene or other suitable fat solvent for a sufficient time for the removal of the fat (usually not less than 1 minute), then drain and dry again. Immerse the slide in 90 per cent ethyl or suitable denatured alcohol for 1 to 2 minutes, dry, and transfer to the staining bath. Avoid the use of old stains or stains containing suspended matter since they may contain bacteria and troublesome precipitates which may cling to the surface of the films. Protect the solvents, fixatives, and stains

when not in use by covering or stoppering the container. Use fresh solutions whenever the old ones contain precipitates or bacteria or are otherwise unfit.

4. STAINING THE FILMS

Prepare the stain by adding 0.3 gm. of certified methylene blue powder to 30 ml. of 95 per cent ethyl or suitable denatured alcohol. Add this solution to 100 ml. of distilled water. Addition of alkali as in the original Loeffler's formula is unnecessary.⁸ Carbolated methylene blue, prepared by adding 10 ml. of a saturated aqueous solution of methylene blue powder to 90 ml. of a 2.5 per cent phenol solution, is preferred by many because it maintains sterile staining solutions.⁹

Dip the slides in the solutions just long enough to stain them properly. Avoid overstaining, if possible. Rinse in water, decolorize in alcohol if overstained, and dry thoroughly before examination. When properly stained or decolorized, the background of the film should show a faint blue tint. Avoid, if possible, the restaining of slides after the application of immersion oil. If immersion oil has been applied, allow the slide to remain in xylene or other fat solvent to remove the oil. Do not use solvents that have been used previously for the removal of immersion oil or for removing fat from the films. Stained preparations may be examined at once or as time permits. The slides may be preserved indefinitely.

The combined solvent, fixing and methylene blue staining solution described by Newman,^{11, 12} known as the Newman-Lampert Formula No. 2, while normally producing neither as clear nor as satisfactory preparations as when the steps are employed separately, is extensively used by technicians in field work because of its convenience and simplicity. Formula No. 2 is as follows:

Methylene blue, certified.....	1 to 1.2 gm.
Ethyl alcohol.....	54 ml.
Tetrachlorethane, technical *.	40 ml.
Acetic acid, glacial.....	6 ml.

Dip the slides in this solution, remove from the stain and *dry thoroughly* before dipping in water to remove the excess stain.

* Listed by Eastman Kodak Company, Rochester, N. Y.

V. COUNTING THE BACTERIA AND GRADING THE MILK

Counting the bacteria, and grading samples of milk with the microscope for official control purposes are two quite distinct procedures which have some features in common. The use to be made of the results and the character of the milk under investigation govern the the type of examination that should be made. When the milk is graded, little attention need be given to making a numerical estimate unless the count approaches the line of class or grade distinction.

Proceed by placing the correct ocular diaphragms or micrometer discs in the proper oculars in order to obtain the field diameters 0.206 mm. (suitable for grading), or 0.146 mm. (preferred for counting).

If the bacteria in a single field measuring 0.206 mm. in diameter are counted, multiply the number found by 300,000 to get the estimated number of bacteria per ml. In practice, more than a single field is examined and the number used is the average found per field. If the bacterial population is small, use preferably a special ocular micrometer disc or suitable ocular diaphragm to reduce the size of the field to 0.146 mm., thus restricting the quantity of defatted solids examined in each field to 1/600,000 part of a ml. of milk or cream. When the examination is limited to the central portion of the field, the use of the margins where the definition of objects is less distinct is avoided and consequently the danger of overlooking bacteria is reduced. Since the magnification used is greater and the field area smaller, count the bacteria in not less than the number of fields, depending upon the number of bacteria found per ml., as hereinafter specified for field diameters of 0.206 mm. and 0.146 mm., respectively. If the field diameter is increased or decreased, count the bacteria in a correspondingly smaller or larger number of fields so that in each instance approximately the same quantity of defatted milk solids is examined.

<i>Range of individual microscopic counts</i>	<i>Number of fields to be examined if the field diameter measures</i>	
	0.206 mm.	0.146 mm.
Under 30,000	60	120
30,000 to 300,000.....	30	60
300,000 to 3,000,000.....	15	30
Over 3,000,000	8	15

PLATE I

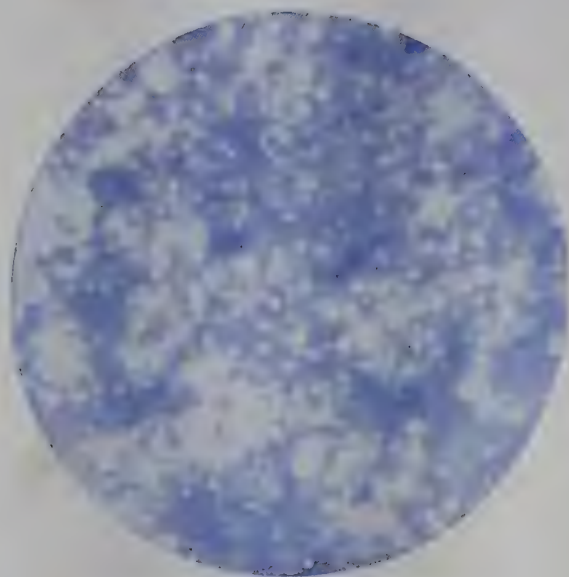


FIGURE 1—When microscopic preparations of clean high-grade milk are stained with methylene blue and examined under a microscope very few if any bacteria can be found. In the preparation shown above the vacuoles left when the fat drops were dissolved show as white circular areas. Magnification 600 \times .

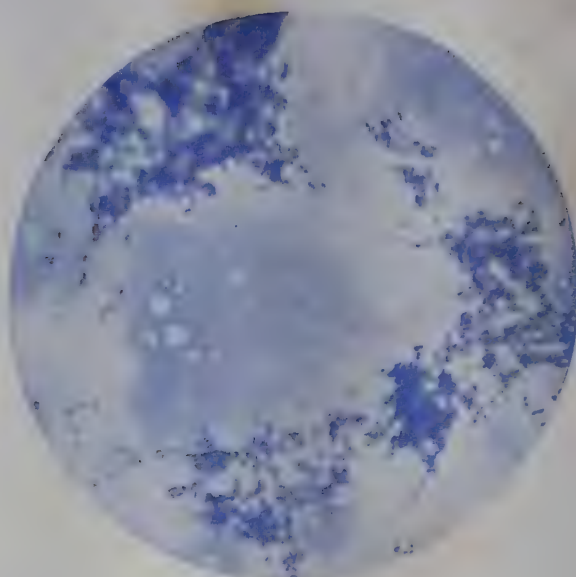


FIGURE 2—When milk is placed in improperly cleaned utensils, it takes up masses of bacteria of miscellaneous types. These are, sometimes micrococci, sometimes non-spore-forming rods, and more rarely large spore-forming rods. Magnification 600 \times .



FIGURE 3—Where high-grade milk is allowed to stand without adequate cooling, the streptococci that cause the normal souring of milk grow rapidly and show as diplococci and as short chains. If preparations are made from buttermilk, they will be found to be present in enormous numbers. Magnification 600 \times .

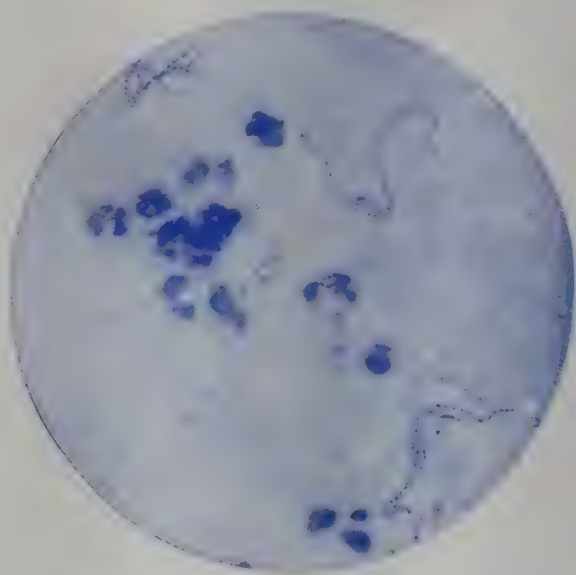


FIGURE 4—Where milk is drawn from the udder of a cow suffering from mastitis (garget), the milk normally contains enormous numbers of leucocytes. In early stages of the infection characteristic long-chain streptococci also occur in large numbers. These characteristic organisms are recognized best in samples taken from individual quarters of the udder or even individual cans of milk. Where infected milk is diluted with larger quantities of good milk, the mastitis streptococci cannot be found as readily. Magnification 600 \times .

In order to permit reëxamination of preparations in case any question arises, preserve microscopic preparations for a reasonable interval after official reports are rendered to the person or persons whose milk has been examined. At intervals as a regular practice, have a second technician check the reports rendered by the first one, particularly in those instances where punitive actions are based on the reports.

When reporting bacterial counts obtained by this method, the same precautions shall be used to avoid giving a fictitious idea of the accuracy of the count as are used when reporting standard plate counts (Section B, VIII, p. 35). Record whether the count represents that of individual bacteria or of clumps of bacteria.

VI. DETECTING CAUSES OF HIGH COUNTS

1. DETECTION OF UDDER INFECTIONS CAUSED BY THE MASTITIS STREPTOCOCCUS

When the object of the examination is to detect the presence of individual cows or quarters of the udder that discharge mastitis streptococci in appreciable numbers, make microscopic preparations in the ordinary way from the proper samples and examine for the presence of long chain streptococci. Obviously the infecting organisms will be found less abundantly as the milk is diluted progressively as by (1) mixing the milk from all the quarters of a single cow, (2) mixing the milk from several cows in individual cans, and (3) mixing the herd milk in the weigh-vat.

If the object of the examination is to detect all infected cows or quarters of the udder, make films from samples of uncooled milk that have been placed promptly in an incubator at 32° C. or 37° C., for 12 to 18 hours. With minimum agitation, transfer a wire loopful of the incubated milk to a slide in order to mount the unbroken chains and clumps of bacteria. Do not use a needle to spread such films. This procedure requires less time than cultural methods for detecting subclinical infections¹³ (Section G, p. 87).

2. DETECTION OF OTHER CAUSES OF HIGH BACTERIAL COUNTS

Four common conditions of raw milk as received in cans at receiving stations are shown in Plate I. Figure 1 on this plate represents the usual appearance of milk where bacteria are too few in number

to be found in ordinary microscopic examination. Figure 2 shows the appearance of the milk where it has become badly contaminated from utensils. Figure 3 shows a milk in which the bacteria present are of the type found in milk that has not been properly cooled. Figure 4 shows the appearance of milk as drawn from a quarter badly infected with the streptococcus of ordinary mastitis.

3. DETECTION OF BACTERIA THAT DO NOT GROW ON AGAR PLATES INCUBATED AT THE USUAL TEMPERATURES

The routine preparation and immediate examination of microscopic preparations from samples of bottled raw and pasteurized milk accomplishes two purposes. If large numbers of stainable bac-



FIGURE XVII—Microscopic appearance of a sample of pasteurized milk at the end of a 5 hour run, showing thermophilic spore forming bacteria. Numerous pin point colonies were developing from the pasteurized milk from this plant on routine plates at this time. 600X. From *New York Agr. Exp. Sta. Bull. 559, 1928.*

teria are found (Figure XVII), remedial measures should be started at once without waiting 48 hours for a Standard Plate Count. This procedure also furnishes the most convenient method of detecting

thermophilic,¹⁴ psychophilic and other types of bacteria, the presence of which may not be indicated by colonies on agar plates incubated at standard temperatures (see Section B, VI, 2, p. 30).

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D. METHYLENE BLUE REDUCTION METHOD

The methylene blue reduction method, frequently called the reductase test,^{1, 2} is based on the fact that the color imparted to milk by a small quantity of a reversibly reducible dye, such as methylene blue, will disappear more or less quickly.^{3, 4, 5} Visual reduction of methylene blue takes place over a narrow oxidation-reduction potential range which is negative to the electro-potential values of fresh, aerated milk.^{6, 7, 8, 9} The evidence is that this negative potential is attained in the incubated milk as a result of the consumption of the dissolved oxygen by growing bacteria. The methylene blue reduction time depends, therefore, on the oxygen consuming power of the bacteria which grow during incubation and, consequently, is indirectly a quantitative index of the bacterial content of the milk at the start of incubation. This relation has been established empirically by the use of the agar plate¹⁰ and microscopic counts¹¹ as well as by keeping quality and other tests.^{4, 5, 12, 13}

I. APPLICATION AND LIMITATIONS

The methylene blue reduction test is most applicable to raw milk. Owing to the small amount of equipment and space required and to the ability of any reasonably intelligent person to operate the test in any milk plant, the method is suited especially to the grading of raw milk supplies where the objective is largely to grade milk samples quickly. Such grades are established arbitrarily by selecting distinctive reduction time intervals as criteria for different grades of milk.

The test has been used extensively as a rapid field test but data are accumulating which indicate that it may be used to improve retail supplies also.^{4, 5, 9, 10, 12, 13, 27} Since it has proved impossible to measure the accuracy of any of the methods of counting bacteria in milk, the sphere of usefulness of the methylene blue reduction test cannot be completely delineated at present.

The main sources of inaccuracy in the test are thought to be⁵:

- a. The inability of some bacteria to grow in milk at 37° C.
- b. The varying influence of different species of bacteria capable of growing in milk at this temperature on the oxidation-reduction

potentials of the milk, presumably because of differing oxygen-consumption rates.

c. Bacteria being swept out of the milk into the cream layer by the rising butter fat.

A modified technic, wherein the tubes are inverted at half-hourly intervals during incubation,⁹ has been adopted in England as the official method for the bacteriological analysis of graded raw milks. In better class milks this technic almost entirely eliminates variability in replicate tubes and uneven disappearance of the dye in the individual tube and usually shortens the reduction time. On this continent opinion is not unanimous regarding the comparative accuracies of the two technics and the effect the modification would have on the usefulness of the reduction test.^{14, 15, 16} A high coefficient of correlation between the standard and modified tests has been reported.¹⁴

The test appears to be increasingly less accurate as the reduction times become longer, and such apparent inaccuracy indicates that no distinctions should be made among milks where reduction times exceed 10 hours. Normal milk drawn aseptically from healthy udders seldom, if ever, reduces methylene blue in less than 10 hours. As milk supplies improve and reduction times lengthen, greater care must be observed in the operation and interpretation of this test.

The sweeping action of the rising butter fat provides an explanation for the common observation that agitation of the milk during incubation usually shortens the reduction time of better-class milk. It is important that the cream layer on the milk in the test tubes be undisturbed after having formed at 37° C. The purpose of agitating the milk (Section D, IV, 1, p. 63), after it has reached the temperature of the water bath is to permit uniform creaming at this temperature.

Methylene blue reduction times are believed to be influenced less by the uneven distribution of bacteria in milk than are agar plate and microscopic counts.^{9, 12} Some bacteria which fail to grow on agar plates undoubtedly function in the reduction test. Exact agreement between reduction times and agar plate counts is not to be expected and for routine milk control work methylene blue reduction times should not be reported in terms of the agar plate count.

Colostrum, mastitis milk, and milk from cows far advanced in

the lactation period may have shorter reduction times than would be expected from their bacterial contents.^{12, 26, 28} In applying the test to herd milks the influence of these abnormal milks is reduced usually to insignificance by successive dilutions.

The reduction time of a very poor milk may be lengthened materially by the usual manipulations consequent upon the practical operation of the test. Therefore, milks reducing in less than 1 hour should not be further differentiated.²²

II. APPARATUS AND MATERIALS

1. PIPETTES, BURETTES AND DIPPER

Measure the quantity of milk to be tested with either a sterile straight-sided one-mark pipette graduated to deliver 10 ml. or a dipper calibrated to deliver 10 ml.⁵ Subject the dipper to practical sterilization as hereinafter described. Use a graduated burette, either mounted or unmounted, or a one-mark pipette graduated to deliver 1 ml. to measure the methylene blue solution into the milk.

2. CULTURE TUBES

Provide sterile culture tubes without lip, preferred dimensions—bore diameter 12 mm. and length not less than 150 mm. Where special tubes of similar diameter and graduated to contain 10 ml. are used, fill the tubes to the mark with any convenient instrument which is sterile or has been subjected to practical sterilization between successive samples. Uniformity of tube diameters is desirable in order to provide for the use of stoppers interchangeably.

Keep the tubes closed with rubber stoppers, except as necessary to open to introduce the sample, in order to protect the contents from contamination during the usual manipulations in collection and incubation. Metal caps for closing the opening of the tube may be used instead of stoppers only when such closures are leak proof when the tubes are inverted.

3. WATER BATHS, INCUBATING CHAMBERS, AND TUBE RACKS

Incubate the tubes of milk at $37^{\circ}\text{C.} \pm 0.5^{\circ}\text{C.}$ either (1) in a water bath of sufficient capacity to bring the samples to 37°C. within an interval of 5 minutes after placing therein or (2) in a thermostatically controlled air incubating chamber as hereinafter described. When an air incubating chamber is used, be sure that (1) the tem-

perature of the samples is 37° C. before placing therein (use tempering bath); (2) observations are made without lowering the temperature of the milk; and (3) the samples are completely protected from light rays including those from the heat source and the pilot light. Do not put the tubes in the water bath in such large numbers at any one time as to delay the warming period more than 5 minutes. Provide a thermostatically controlled heat source for the water bath. To insure uniformity of temperature in baths, it may be necessary to install a means to circulate the water continuously.

During the test avoid exposure to direct sunlight and to strong electric light because these are known to catalyze the reduction of the methylene blue in the milk.^{8, 23, 24, 25} Protect the tubes from strong light if it is necessary to incubate them in a window, beneath a skylight, in the direct path of rays from the sun, or within 6 ft. of an electric light. All-glass or glass-front water baths should be painted or protected in some way to prevent transmission of light through the glass. Do not use electric light bulbs immersed in the water unless the tubes are completely protected from the rays of light.

Provide rust-resistant sheet metal or wire racks for arrangement of the test tubes in single or double rows, and so constructed that each tube is held in a vertical position in the water bath or incubator to insure at all times against disturbance of the contents of the tubes.

4. METHYLENE BLUE TABLETS AND SOLUTION

In the original European methylene blue reduction technic Barthel and Orla-Jensen standardized the methylene blue solution by suitable aqueous dilutions of a saturated alcoholic solution of the dye but later recommended the use of tablets containing a constant quantity of the dye. Such tablets, supplied by Blauenfeldt and Tvede, Copenhagen, to take the place of the less satisfactory solutions, were used as a guide in the preparation of the first American tablets.

In the past the dye in the European and American tablets has been methylene blue chloride. Because of the difficulty of purifying and standardizing methylene blue chloride, the dye in the present American tablets is methylene blue thiocyanate which is readily prepared in a state of practical purity and is, therefore, reproducible.

Methylene blue chloride and methylene blue thiocyanate in equal concentrations have identical reaction values in the reduction test.^{17, 21} The tablets are manufactured from certified methylene blue thiocyanate and are sold through all the usual laboratory supply houses. Use of the older methylene blue (methylene blue chloride) tablets is no longer an approved procedure.

The early European technic resulted in a concentration of approximately 1 part of methylene blue to 300,000 parts of milk, while the American and later European²⁰ procedures have resulted in concentrations considerably weaker than this.¹⁸ The proper concentration is the weakest which will give a sharp end point of reduction. A concentration of 1 part of dye to 300,000 parts of milk gives satisfactory results when the procedure outlined below is followed. This is a stronger concentration than was obtained with the former American methylene blue chloride tablets and will lengthen somewhat^{19, 20, 21} the average reduction time of milk. This increased reduction time is due to increased concentration of the dye and not to substitution of methylene blue thiocyanate for methylene blue chloride.

Use only the methylene blue thiocyanate tablets which have been certified by the Commission on Standardization of Biological Stains to be suitable for the methylene blue reduction method. Dissolve one tablet in 200 ml. of sterile or freshly boiled distilled water (not from a steam line condensate), observing that the volume of the solution at room temperature is 200 ml. Solution is complete when one tablet is allowed to stand in 200 ml. of water over night.

Always protect the aqueous solution of the dye from light to prevent its rapid deterioration. Store the dye in amber glass bottles in the dark. In practice, a fresh solution is usually prepared weekly to avoid the problem of deterioration.

III. COLLECTION OF SAMPLES

Collect sample as directed under the Agar Plate Method (Section A, pp. 7-13), or with a 10 ml. dipper transfer 10 ml. of milk directly from the weigh tank at receiving stations into sterile test tubes in which the test is to be made; or, in cases in which the milk is dumped from cans directly into the pasteurizing vat, collect in a sterile container 10 ml. portions of milk from an equal number of cans of night and morning milk, and immediately after thorough mixing remove the 10 ml. samples from this composite.

If a 10 ml. dipper is used, rinse it immediately after sampling in clean cold running water and place it in water at not less than 180° F. until the next sample is taken. If samples are taken with pipettes, use pipettes which are sterile or have been subjected to practical sterilization measures (Section A, IV, p. 9). Do not use chlorine or other chemical solutions as sterilizing agents because residues therefrom may influence the test.

Store tubes containing the collected samples in ice water for a period not to exceed 2 hours before starting their incubation in the water bath. This storage interval is for the convenience of assembling either a full rack of tubes or the complete set of samples so that the incubation of several samples can be started at one time. The practice of storing the samples for more than 2 hours before starting the test is discouraged because of the difficulty of redistributing the more densely formed cream layer uniformly throughout the samples when mixing with the dye.

IV. DESCRIPTION OF TEST

• 1. PREPARATION AND SETTING THE TEST

Use glassware which is scrupulously clean and free from etching to prevent precipitation of the methylene blue salt. Cleaning solutions may be used (Section B, IV, 6, p. 29). If possible, sterilize the containers for the dye solution, the pipettes, test tubes, and rubber stoppers in an autoclave. If impossible, boil the glassware and stoppers immediately before use and allow the retained heat to dry the equipment. Avoid recontamination of that portion of the sterilized stopper which is inserted into the test tube.

With a burette or a 1 ml. pipette, add 1 ml. of the methylene blue thiocyanate solution to the tube either a short time before or after the milk has been placed therein. Immediately before or after the addition of the sample, identify each tube legibly and indelibly with the producer's name or number (1) by writing on the tube, (2) by means of an appropriate chart, or (3) in some other suitable manner. Close or stopper the tube as soon as the sample is added. When ready to start a batch of tubes, transfer them from the ice water or other refrigerant to a water bath which will bring them to a temperature of 37° C. within an interval of 5 minutes. When the contents of the tubes have reached a temperature of 37° C. invert the tubes a few times to assure uniform creaming, after which avoid

agitation which would disturb the cream layer.^{5, 22} Incubate the tubes at $37^{\circ}\text{C.} \pm 0.5^{\circ}\text{C.}$ either in a water bath or in an air chamber.

When practical, avoid placing samples in the water bath in such large numbers that they will not reach a temperature of 37°C. within an interval of 5 minutes. If the number of samples or their temperature when placed in the tempering bath is such as to require a tempering period exceeding 5 minutes, preheat the samples after the milk is placed in the tubes in an auxiliary water bath controlled at a temperature not exceeding 40°C. The use of a larger volume of tempering fluid or of forced agitation of the medium at the higher temperature will increase the rate of heat transfer. Determine the temperature of the milk in the tubes by immersing the bulb of a thermometer in a blank tube containing milk which has been treated identically with the other tubes in the group to be subjected to the test.

2. REDUCTION TIME AND END POINT

The methylene blue reduction time is the interval between the placing of the tubes in the 37°C. water bath or incubator, immediately after their inversion, and the nearly complete disappearance of the blue color from the milk. An interval timer or an alarm clock may be used to inform the observer when the first and each successive examination of the tubes is to be made.

The exact end point of reduction is not always easily determined as many of the better class milks reduce unevenly throughout the tube. In certain European countries the color in the upper fourth is neglected in reading the end point of reduction. The objection to this procedure is that when the color persists in certain portions of the milk it is not invariably in the upper portion. It is preferable to consider reduction complete when four-fifths of the visible portion of the contents of the tube have turned white. In better class milks the end point is seldom sharp enough to permit accurate recognition at intervals of less than 15 minutes.

3. SUGGESTED GRADES AND CLASSES

Within the limits of 1 and 10 hours, any classification of milk based on the methylene blue reduction test is necessarily an arbitrary

one. A herd milk that reduces in 2 hours or less undoubtedly has a high bacterial content. One that requires 8 hours for reduction probably contains comparatively few bacteria other than those in the milk at the time of its withdrawal from the udder. The following classification is presented merely as a possible guide. This classification is not intended to carry with it the inference that all milk that decolorizes in less than 8 hours is unacceptable for use as market milk.

Class 1. Excellent, not decolorized in 8 hours

Class 2. Good, decolorized in less than 8 hours but not less than 6 hours

Class 3. Fair, decolorized in less than 6 hours but not less than 2 hours

Class 4. Poor, decolorized in less than 2 hours

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V. APPENDIX. THE RESAZURIN TEST

In 1928 Pesch and Simmert¹² proposed the substitution of resazurin for methylene blue in the reduction test, the reduction being carried to the pink end point. It was erroneously believed that the irreversibility of this reaction renders it insensitive to the oxygen dissolved in the milk. Johns,⁸ and Collins et al.¹ preferred this test because results are secured in a shorter time than results from the methylene blue reduction test. They did not agree on the extent of shortening. Russell et al.¹⁴ feel that this test responds to the presence of abnormal milk better than does the methylene blue test.

Ramsdell et al.,¹³ developed a resazurin test in which time is constant and color is the variable, the color at the end of 1 hour of incubation indicating the bacteriological condition of the milk. Davis suggested two other tests, the 5 minute resazurin test³ and the resazurin-rennet test.⁴ The chemistry of resazurin,¹⁵ the suitability of the commercial preparations now on the market,^{5, 11, 15} end points,⁹ anomalies^{2, 6, 7, 10, 15} and a suitable resazurin test are all at present in controversy.

In view of this situation, no recommendation either for or against the use of this test is made at the present time.

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E. SEDIMENT TEST

Because much of the dirt that appears as a visible and insoluble sediment in milk is accompanied by relatively few bacteria in proportion to the number derived from other sources, it frequently becomes desirable to use a test that will reveal the amount of visible sediment in the milk. For this reason, a description of the commonly used sediment test has been included here.¹ By using this test valuable information may be obtained and the occasional approval of a dirty milk prevented.

With the development of efficient single service strainers for use on dairy farms, and efficient strainers, filters, and clarifiers for use in milk receiving plants, the sediment test is becoming more and more a measure of the efficiency of these sediment removing devices. In most cases, it is necessary to visit the farm during the milking operation in order to determine whether the product delivered is "clean" or "cleaned" milk.

In utilizing the results of sediment tests it should always be borne in mind that the sediment test does not yield results which compare in accuracy with the accuracy of many other analytical procedures. The sediment which may be found in milk in different regions may vary considerably in composition, specific gravity, color, and other physical characteristics so that an equivalent weight of sediment in one section may appear considerably different from that in another section. For this reason any one particular sediment standard may fall short of the perfection desirable in a satisfactory laboratory standard. If the limitations of the test are kept in mind the quantitative sediment test can, however, be made to serve a very useful purpose.

I. SAMPLES

For bottled milk sold at retail, pint samples only are regarded as standard. Where quart or any other size sample is used, state on the report the size or type of sample used. Samples may be taken from pint or well shaken quart bottles of milk.

In many cases effective sediment testing of milk received at the receiving platform may be carried on by use of the "off-the-bottom"

method of testing. In this procedure the sediment is collected from the bottom of an unstirred can of milk with a sediment tester* designed for this purpose. Testers of this nature should be as simple as possible in character in order that they may be kept clean to prevent the possible contamination of milk which is being sampled. At least one half pint of milk and where possible a pint of milk should be withdrawn from the bottom of the can for filtration purposes.

Reports showing results of off-the-bottom testing shall indicate that the test is made from a bottom sample and not from a thoroughly mixed can. Since the procedure is essentially qualitative rather than quantitative it will be necessary for local enforcement officials to determine the amount of sediment which will be unacceptable by this procedure. For this reason any acceptable sediment standard chart may be used and the limiting values on this standard chart may be established to meet local conditions. More dirt will be visible on the sediment discs when this procedure is used than when the standard quantitative procedure is used.

Discs showing sediment may be shown to producers. Such showings usually readily convince producers that it is desirable to use cleaner methods of production.

* The Hinman testers sold by the Hinman Milking Machine Company, Oneida, N. Y., and the Carkhuff tester sold by E. B. Carkhuff, Binghamton, N. Y., are being used in a number of places. A tester marketed by the Rapid Tester Corporation, Milwaukee, Wis., has also been used for carrying out this procedure.

II. SEDIMENT DISCS

The pint samples of milk shall be strained through firm* cotton discs² placed over openings 1 in. in diameter. It will hasten the process of filtering if provision is made for warming the milk, or the milk may be forced through the disc by air pressure. With certain of the off-the-bottom testers special types of sediment discs are required which can be furnished by the company marketing the tester.

There is considerable variation in the ease with which different lots of milk can be filtered, pasteurized milk more frequently giving trouble than raw milk. One of the causes of difficult filtering is apparently a physical change in the fat, which results in small clumps

* Of the type furnished by the Lorenz Model Co., Madison, Wis., makers of the Wisconsin Sediment Tester.

of fat globules being present, but there are undoubtedly other factors also. A partial coagulation of the proteins in the milk, due to high temperatures, has been suggested as a cause of the stopping of the filter with certain lots of pasteurized milk.

III. PREPARATION OF STANDARD SEDIMENT DISCS

Standard discs showing definite amounts of sediment are useful in order to make comparisons. Various types of dirt have been used in preparing these discs. The Connecticut standards mentioned below, for example, are prepared from mixed stable dirt of standard fineness to which has been added approximately 20 per cent of sand fine enough to pass a 100 mesh sieve.³

The following directions for preparing standard discs⁴ are given by Hoffmann:

A suspension of weathered, dried, finely ground cow dung is first prepared, a 50 per cent cane sugar solution being used as the medium.

The cow dung is dried in an oven and ground through a laboratory feed mill several times. Practically all the ground material should be fine enough to pass a 60 mesh screen, and the greater portion should be finer than 100 mesh. One-tenth gm. is accurately weighed and transferred to a 1,000 ml. measuring flask, the 50 per cent sugar solution also being used to wash all the fine particles down into the flask. The volume is made up to the 1,000 ml. mark with more 50 per cent sugar solution after most of the fine particles have been wetted by shaking the half-filled flask thoroughly several times. After the volume is made up to the mark, the contents of the flask are shaken vigorously every 5 minutes for sufficient time to saturate the particles thoroughly ($\frac{1}{2}$ to 1 hour). When the particles have been thoroughly wetted it will be noted that the sugar solution will hold them very evenly in suspension, and the mixture is now ready to use in making the standard discs.

On the basis of 0.1 gm. per 1,000 ml., 10 ml. of the sugar solution contains 1 mg. of sediment. Test discs are made with one of the sediment testers commonly in use, varying volumes of the sediment solution being used. Several ounces of filtered skim milk are placed in the sediment tester and varying volumes of the sediment solution are added. After forcing the milk through the discs, a small quantity of filtered skim milk is run through. By using a small quantity of milk in the tester at the time the sediment solution is added, a more even distribution of the sediment on the

disc is obtained. The purpose of following through with more skim milk is to be sure that all the fine particles are washed onto the disc, and also to obtain an adhesive condition that will make the sediment adhere to the disc.

Adding the sediment-sugar solution directly to a small volume of milk and filtering the entire amount does away with a possibility of any error entering into the results due to poor sampling. The disc is then removed from the tester, mounted permanently on a stiff paper, allowed to dry, and then made permanent by spraying with a strong disinfectant such as corrosive sublimate. A good apparatus for this purpose is an ordinary throat atomizer, provided caution is observed not to use corrosive sublimate in contact with metal. Below each mounted standard disc on the paper should be noted the quantity of dried material that the dirt or filth on the disc represents. If these standards are to be handled or are to be used for any length of time they should be placed under glass.

IV. PHOTOGRAPHIC STANDARDS

For those who do not wish to prepare their own discs, copies of the photographic standards prepared by the Connecticut State Department of Health Laboratory may be secured through the office of the American Public Health Association, 1790 Broadway, New York, N. Y., at a cost of \$1.00 each for the 1931 standard and \$0.60 for the 1939 standard. The standards on this photograph are based on pint samples of milk to which weighed amounts of sediment have been added. The 1931 standards cover the range from clean to very dirty milk with numerical ratings or sediment scores given for each disc. The 1939 standards, which are designed chiefly for use with stirred samples of milk, show only three sediment discs and grade the milk into four cleanliness grades.

In using these standards each sediment disc should be graded to the nearest sediment score whether actually above or below the photographic standards. No attempt should be made to grade as sediment any hair, pieces of hay or straw or large particles of dirt. These should be reported separately and in addition to the amount of sediment which compares with the photographic standard.

V. METHODS OF USING DISCS

After recording the "sediment score" or "cleanliness rating"

from each disc, the discs may be prepared (1) for return by mail to dairymen or milk dealers, (2) for posting on a bulletin board at a milk plant, or (3) for filing. This may be done by drying the discs and placing them in transparent waxed paper envelopes slightly larger than the discs. The waxed paper envelopes with the contained discs may be mailed in ordinary envelopes. Where the discs are to be posted on a bulletin board or attached to cards for filing, they may be placed on these while still moist with milk, and allowed to dry. The dried milk serves as an excellent glue. If the discs become too dry to stick to the cardboards, moisten with a few drops of water. Ordinary glue is not satisfactory for this purpose.

VI. REPORTS

Experience has shown that one of the most effective ways of reporting results to dairymen or dealers is to paste the discs on a bulletin board or to return a duplicate disc to them with a statement as to the way in which it was obtained. The commonest criticism that can be applied to the comments sent with such reports is that they frequently make claims regarding the influence of dirt upon the bacterial count which are not substantiated by investigation.^{5, 6, 7} Dirt of any sort is sufficiently objectionable in milk or any other food to be condemned solely on the ground that it is dirt; and it only weakens the objection to attempt to bolster it by doubtful claims as to an exact correlation between bacteria and dirt. The general effect of the use of the sediment test has usually been to cause a noticeable reduction in the amount of visible sediment.

VII. VISIBLE SEDIMENT IN BOTTLED MILK

When bottled milk has been allowed to remain in a quiescent state small amounts of sediment may be noted in the bottom of the bottle. The presence of any visible sediment in bottled milk when viewed in this manner is undesirable. For rapid inspection purposes bottled milk showing the presence of visible sediment may be considered as having a sediment score of at least 50.

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F. TESTS FOR ORGANISMS OF THE COLIFORM (ESCHERICHIA-AEROBACTER) GROUP

I. INTRODUCTION

These tests may be employed for various purposes. Since small numbers of the coliform group of organisms may enter milk or cream under normal conditions of production and handling, and since multiplication of such organisms in milk is favored by the same conditions that favor multiplication of other bacteria, discretion must be observed in the application of this determination to the specific purpose intended and particularly in the interpretation of the results obtained.

The following observations with regard to the significance of the occurrence of organisms of the coliform group in milk or cream may be noted:

1. In general, the greater the care employed in the production and the handling of milk or cream, the smaller will be the number of coliform organisms which it contains. This relationship parallels that which usually obtains between the degree of sanitation employed and the agar plate count. Because of this fact, many workers regard the coliform group test, when applied to miscellaneous samples of milk, as an unnecessary duplication of effort.

2. *Raw Milk*—Since certain organisms of the group are known to occur occasionally in milk from inflamed udders, their presence in fresh milk may sometimes indicate an udder infection. Organisms of the coliform group are frequent contaminants of poorly sterilized utensils, such as milk pails, milking machines, cans, etc. These bacteria multiply rapidly when environmental conditions are favorable. In consequence, even when a raw milk is tested within 3 to 4 hours after production, additional tests of samples drawn aseptically from the udder and of samples obtained by rinsing utensils, strainer-cloths, udders, etc., in sterile water are necessary in order to determine the immediate source of any contamination with organisms of this group.

3. *Growth*—Unless a raw milk is tested within 3 to 4 hours after production, or has been produced and cooled under such satisfactory conditions that the total count is low, *i.e.*, less than 10,000

farmml.,* growth normally plays such a part it becomes impossible to determine the significance of the results secured.^{1, 2, 3} It is likewise impossible to determine the significance of results secured from samples of pasteurized milk which have been stored at temperatures that permit growth.

4. *Pasteurized Milk*—Organisms of the coliform group are practically eliminated from milk and cream by proper commercial pasteurization procedures.^{4, 5} For this reason, where 1 ml. samples of freshly pasteurized milk give positive results from the bottled milk, either improper pasteurization or contamination of the pasteurized product by organisms of the coliform group growing on or in the equipment or in the final container may be suspected.

* The American Association of Medical Milk Commissions has fixed a standard of less than 10 coliform organisms per ml. for certified milk. See *Methods and Standards for the Production of Certified Milk*. National Headquarters, 1265 Broadway, New York, N. Y., 1940.

II. DEFINITIONS

1. *Coliform Group*⁶—It is recommended that the coliform group be considered as including all aerobic and facultative anaerobic, Gram-negative, non-spore-forming bacteria which ferment lactose with gas formation.

2. *Presumptive Tests*—The formation of gas within 48 hours at 37° C., in a fermentation tube containing Brilliant Green Bile (2 per cent)^{4, 13} or Formate Ricinoleate Broth^{7, 8} is presumptive evidence of the presence of organisms of the coliform group.

Favorable experience has been reported of the use of direct plating media such as Violet Red Bile Agar^{9, 10, 11, 13} and Desoxycholate Agar.¹² The appearance in the agar of typical dark-red colonies of at least 1 mm. in diameter may be considered presumptive evidence of the presence of coliform organisms.

3. *Completed Tests*—To complete the demonstration of the presence of organisms of this group, it is necessary to show that the fermentation tubes, in which gas has appeared, or the typical colonies appearing in the direct plating agar media, contain Gram-negative, non-spore-forming bacilli which, when inoculated into a lactose broth fermentation tube, form gas within 48 hours upon incubation at 37° C.

III. APPLICATIONS

1. *Pasteurized Milk and Cream*—Since 10 ml. volumes of prop-

erly pasteurized milk or at least 1 ml. volumes of properly pasteurized cream very rarely contain organisms which ferment Brilliant Green Bile (2 per cent) or Formate Ricinoleate Broth, or which produce typical colonies in Violet Red Bile Agar or Desoxycholate Agar, it is recommended that the standard test for coliform group organisms in routine pasteurization control work be limited to the Presumptive Test.

2. *Raw Milk and Cream*—It is recommended that the Presumptive Test be also employed when the coliform group test is applied to raw milk or raw cream control, but that the Completed Test be used from time to time to confirm the applicability of the Presumptive Test to the samples examined, for some organisms which are not of the coliform group produce gas in the liquid media or typical colonies in the agar media.

3. *Homogenized Milk*—In industrial practice milk is usually preheated to a temperature of about 145° F. to facilitate homogenization. Homogenized pasteurized milk should conform to the same standards as pasteurized milk. It is recommended that the standard test for coliform group organisms in routine control work on homogenized milks be limited to the Presumptive Test.

4. *Cultured Milk*—The preparation of cultured milks (buttermilk, etc.) involves incubation at temperatures that are more or less favorable to the multiplication of pathogens which may be contained in the original milk or which may be introduced into it as contaminants. Although the low pH attained by certain types of cultured milk offers a measure of safety from the danger of such contamination, the hazard of incubation of pathogens that may be contained in such products is so great that all cultured milks should conform to the same standard as pasteurized milk. It is recommended that the standard test for coliform group organisms in routine control work on cultured milks be limited to the Presumptive Test.

IV. TYPE OF SAMPLES

Eliminate uncertainties regarding the nature and source of the organisms by the examination of samples taken under controlled conditions. Of raw milk supplies other than certified, samples shall be taken from individual cans or bottles of milk not more than 3 to 4 hours old. When continued positive Presumptive Tests are secured in 0.1 ml. samples of raw milk, visits shall be made to the

farms and samples secured aseptically from individual cows, from rinsings of utensils with sterile water, etc. In this way, the probable source and significance of the presence of these organisms may be determined.

When positive Presumptive Tests are secured from samples of pasteurized milk secured as the milk leaves the pasteurizer, examine additional samples in conjunction with inspections in order to determine why the organisms of the coliform group are not killed during pasteurization. Check samples of this type with the Phosphatase Test to determine the efficiency of pasteurization in all cases.

When positive Presumptive Tests are secured from bottled pasteurized milk, samples shall be secured at the pasteurizing plant. These shall be taken from the milk as it leaves the pasteurizer and from such other places as may be necessary to determine the exact source of the contamination with the organisms of this group. It is especially advisable to sample, at various points, the first milk passing through the equipment; for such samples, containing the first washings of crevices where coliform organisms may have been multiplying between runs, particularly favor the detection of defects of this character. Furthermore, the contrast between the results secured from samples taken at the beginning and end of the pasteurizing run may furnish information of value.

V. MATERIALS

1. BRILLIANT GREEN LACTOSE PEPTONE BILE (2 Per Cent)

This medium contains brilliant green in a concentration of 1 : 75,000 and 2 per cent of dehydrated bile.

Dissolve 10 gm. of peptone and 10 gm. of lactose in not more than 500 ml. of distilled water. Add 200 ml. of fresh ox bile or 20 gm. of dehydrated ox bile dissolved in 200 ml. of distilled water. No dehydrated ox bile shall be used which has a pH of less than 7.0. Make up with distilled water to a total of at least 975 ml. and adjust the reaction to a pH of 7.4. Add 13.3 ml. of a 0.1 per cent solution of brilliant green (certified dye) in water; make up to a total of 1,000 ml. and filter through cotton.

Distribute the medium, in tubes provided with inverted vials, in 10 ml. quantities for tests of 1 ml. or less of sample. If 10 ml. of

sample are to be tested, the medium shall contain one and one-half times the proportions of the various ingredients indicated above, and shall be tubed in 20 ml. quantities.

Sterilize the tubes of medium in the autoclave at 15 lbs. pressure (121° C.) for 15 minutes.

The reaction after sterilization shall not be less than pH 7.1 and shall not be more than pH 7.4. This reaction shall ordinarily be determined by electrometric methods using the hydrogen or glass electrode although it is reported that colorimetric methods may be used with satisfaction before the addition of brilliant green if the medium is diluted 1:4 with distilled water and phenol red is selected as the indicator.

The above medium may be used in the dehydrated form, in the proportion of 40 gm. of the dehydrated material to 1,000 ml. of distilled water when 1 ml. or less of the milk or cream sample is tested; and in the proportion of 60 gm. to 1,000 ml. of distilled water, tubed in 20 ml. quantities, when 10 ml. of sample is tested.

2. FORMATE RICINOLEATE BROTH

Add 5 gm. of peptone, 5 gm. of lactose, 5 gm. of sodium formate, and 1 gm. of sodium ricinoleate to 1,000 ml. of distilled water. Heat slowly on a water bath with constant stirring, until ingredients are dissolved. Add distilled water to make the volume 1,000 ml. Adjust the reaction so that the pH reading after sterilization will be 7.3–7.5.

Distribute the medium, in tubes provided with inverted vials, in 10 ml. quantities for tests of 1 ml. or less of sample. If 10 ml. of sample are to be tested, the medium shall contain one and one-half times the proportions of the various ingredients indicated above, and shall be tubed in 20 ml. quantities.

Sterilize the tubes of medium in the autoclave for 15 minutes (not longer) at 15 lbs. pressure (121° C.).

The above medium may be used in the dehydrated form, in the proportion of 16 gm. to 1,000 ml. of distilled water when 1 ml. or less of the milk or cream sample is tested; and in the proportion of 24 gm. to 1,000 ml. of distilled water when 10 ml. of sample is tested. Tube in 10 ml. or in 20 ml. quantities as directed above.

3. VIOLET RED BILE AGAR * AND DESOXYCHOLATE AGAR †

These media may be prepared from the various ingredients indicated in published formulae, but in the interest of uniformity it is recommended that they be used in the dehydrated form, in the proportions of 41 gm. of dehydrated Violet Red Bile Agar to 1,000 ml. of distilled water and of 46 gm. of dehydrated Desoxycholate Agar to 1,000 ml. of distilled water. Sterilize the first of these agar media in the autoclave for 15 minutes at a pressure of 15 lbs. (121° C.); the second in flowing steam for 30 minutes.

* Obtainable from Difco Laboratories, Detroit, Mich.

† Obtainable from Baltimore Biological Laboratory, Baltimore, Md.

VI. MAKING THE TEST

1. THE PRESUMPTIVE TEST

a. Liquid Media—Inoculate a series (5 tubes of each dilution used are recommended) of Brilliant Green Bile (2 per cent) or of Formate Ricinoleate Broth fermentation tubes with appropriate graduated quantities (decimal multiples or fractions of 1 ml., such as 10 ml., 1 ml., 0.1 ml., 0.01 ml., etc.) of the milk to be tested. In order to be certain of obtaining a definite result, it is essential that the dilutions be such that at least one positive and one negative tube result be obtained. To satisfy this requirement, it may be necessary to plant three or even more dilutions.

When, however, the purpose of the test is merely to determine whether a specific density of organisms is exceeded, only one or two dilutions may be required. In pasteurization control, for example, 5 tubes or even 3 tubes each inoculated with 1 ml. (or 10 ml.) are sufficient where frequent routine analyses have shown that results are likely to be negative. In this work as in bacterial count work, a simple examination of routine samples taken frequently will give more useful information than a more complete examination of samples taken at infrequent intervals.

Incubate these tubes at 37° C. for 48 hours. The formation, within this period, of gas constitutes a positive Presumptive Test.

b. Solid Media—Place not more than 1 ml. of sample in a 100 mm. × 15 mm. Petri dish. Add 10 to 15 ml. of Violet Red Bile Agar, or Desoxycholate Agar, which has been liquefied and cooled to a temperature of 40° to 44° C., and mix thoroughly by tilting and rotating the dish.

After the agar and sample mixture has solidified, 3 or 4 ml

of agar are poured over it to form a film of medium which covers the entire surface of the solid mixture. The purpose of the cover is to eliminate the possibility of the occurrence of surface colonies of coliform organisms, for the appearance presented by such colonies is often so atypical that they may not readily be recognized.

When the agar cover has solidified, the plates are placed in an inverted position in the 37° C. incubator for a period of 20–24 hours.

The appearance, at this time, of typical dark-red colonies of at least 0.5 mm. in diameter constitutes a positive presumptive test. These colonies should be counted and the number recorded.

2. THE COMPLETED TEST

a. From Positive Fermentation Tubes—Streak an eosin methylene blue or Endo agar plate from the fermentation tube. It is advisable to make the transfer as soon as possible after gas formation in the tube has occurred, and so to distribute the material over the plate as to insure the presence, upon incubation, of discrete colonies. Incubate the plate at 37° C. for 18 to 24 hours.

From the plate fish one or more typical colonies, or if no typical colonies are present, two or more colonies considered most likely to be of organisms of the coliform group, and transfer each to a nutrient agar slant and to a lactose broth fermentation tube. The agar slant is incubated at 37° C. for 24 hours and the lactose broth tube for 48 hours. The formation of gas in the lactose broth and the demonstration, upon microscopical examination of a Gram-stained preparation of the agar slant culture, of the presence of Gram-negative, non-spore-forming, rod-shaped bacteria, and of the absence of spore-forming bacilli, constitute a positive Completed Test. If these conditions are not satisfied, the result is negative. It may be desirable, in special cases, to undertake a further study of the agar culture in the effort to isolate an organism which will satisfy these conditions.

b. From Positive Selective Agar Plates—Although selective agar media inhibit the formation of visible colonies by many of the milk or cream bacteria which are not of the coliform group, these bacteria may not all be killed by the inhibitory agents. As a consequence, material fished from a deep typical colony may consist of a mixed culture of organisms from the colony and organisms from the adjacent medium; purification of the colony culture is therefore necessary.

Such purification, for routine work, is effected by transferring material from the typical dark-red colony to a lactose broth fermentation tube which is incubated at 37° C. As soon as possible after gas appears, the complete procedure described in (a) above should be followed. If no gas is formed within 48 hours, the colony fished must be considered to have contained no organisms of the coliform group.

VII. EXPRESSION OF RESULTS

Results shall be recorded as observed, indicating the amount tested and the result from each.

VIII. THE COMPARATIVE PRECISIONS OF THE TUBE AND PLATE METHODS ¹³

An agar plate count of 4 or 5 colonies or more, is a result quite as precise, in the long run, as the result obtained from the use of 10 fermentation tubes. But when fewer coliform organisms are present, completely negative results may occur with a frequency which renders the single-plate method of little utility. For example, even when the sample contains 1 coliform organism per ml., about 37 per cent of 1 ml. plates may be expected to yield negative results, because of irregular distribution of the bacteria in the sample. When 5 tubes, with 1 ml. of sample in each, are employed under these conditions, a completely negative result may be expected less than 1 per cent of the time. It is evident, therefore, that if counts of at least 4 typical colonies can be expected, the single-plate method may prove as convenient as the usual 5-tube method and yield results of greater precision than those afforded by the tubes; but if counts of less than 4 colonies are to be expected, the labor of pouring two or more plates (in order to increase the total count) may offset the advantage of the greater precision of the plate results, and the simpler tube procedure, involving the use of several fermentation tubes, may be preferred. When large volumes of sample, such as 10 ml. are to be tested, the use of tubes is almost indispensable.

Even when 5 fermentation tubes are employed, however, the precision of the result obtained is not of a high order. The following table shows the significance to be attached, with a reasonable degree of assurance, to results obtained by the use of 5 tubes with one dilution of the sample.

The probability of 97 per cent is taken as the criterion, signifying

that, on the average, the range of numbers of organisms will be as shown in about 32 out of 33 samples yielding the result indicated. These odds are not very great but they provide a fair degree of assurance.

Case: 1 ml. of sample in each of 5 tubes.

Result in number of positives	Most probable number per 100 ml.	Probability is 97 per cent that the number of organisms is within the range
0	0	per 100 ml. 0-70
1	22	4-140
2	51	12-210
3	92	29-320
4	161	53-580

If 10 ml. are placed in each tube, instead of 1 ml., the figures given above should be divided by 10; if 0.1 or 0.01 ml. quantities are placed in each tube, the figures should be multiplied by 10, 100, etc.

Thus, excluding from consideration all errors except that of chance distribution of organisms in the well shaken sample, a result of 5 negative tubes (each inoculated with 1 ml. of sample) provides fair assurance that the sample does not contain more than about 70 organisms per 100 ml. of sample. It is obvious that, because of the large error of simple sampling which obtains under these conditions of testing, considerable caution must be exercised in drawing conclusions from the results of tests of one sample, even when 5 tubes are used.

IX. PROBABLE NUMBER OF ORGANISMS PER 100 ML.^{14, 15, 16, 17}

It is frequently convenient to report, also, the probable number of organisms per 100 ml. of sample, indicated by the result of the fermentation tube tests. Since more than one organism may be responsible for a positive fermentation tube result, the probable number of organisms is a logarithmic function of the tube result. The accompanying table gives the probable numbers corresponding to results obtained from the 5-tube system of examination, when one dilution, two dilutions, and three dilutions are employed.

This table is calculated for results obtained from 5 tubes with

each dilution, beginning with the dilution 10 ml. If the dilutions employed start with 1 ml., the probable number given by the table should be multiplied by 10. Thus the results $\frac{1 \text{ ml.}}{3+}$ $\frac{0.1 \text{ ml.}}{1+}$ may be entered in the table as $\frac{10 \text{ ml.}}{3+}$ $\frac{1 \text{ ml.}}{1+}$ and the corresponding probable number, 11 per 100 ml. is multiplied by 10 to give 110 per 100 ml., as the final probable number of organisms of the group in the sample. Similarly, if the largest dilution is 0.01 the probable number given by the table is to be multiplied by 1,000.

When more than three dilutions are employed, the results from only three are significant. To choose these the highest $\frac{5}{5}$ below which no smaller result occurs, should be taken, and also the two following. In the following examples the significant dilution results are italicized:

	1 ml.	0.1 ml.	0.01 ml.	0.001 ml.
a)	$\frac{5}{5}$	$\frac{5}{5}$	$\frac{2}{5}$	$\frac{0}{5}$
b)	$\frac{5}{5}$	$\frac{4}{5}$	$\frac{2}{5}$	$\frac{0}{5}$
c)	$\frac{0}{5}$	$\frac{1}{5}$	$\frac{0}{5}$	$\frac{0}{5}$

In the example (c) above, the first three dilutions should be taken, so as to throw the positive result in the middle dilution.

When a case such as the following (d) arises, where a positive occurs in a dilution higher than the three chosen according to the rule, it should be included in the result of the highest chosen dilution, making the result read as in example (e).

	1 ml.	0.1 ml.	0.01 ml.	0.001 ml.
d)	$\frac{5}{5}$	$\frac{3}{5}$	$\frac{1}{5}$	$\frac{1}{5}$
e)	$\frac{5}{5}$	$\frac{3}{5}$	$\frac{2}{5}$	$\frac{0}{5}$

When all tubes are positive, a minimum probable number may still be recorded by considering the number of tubes planted with the smallest amount of sample to have been 6 instead of 5. In

TABLE OF MOST PROBABLE NUMBERS USING FIVE TUBES WITH EACH DILUTION¹⁶
More complete tables will be found in the Standard Methods of
Water Analysis, 8th Ed., 1936, pp. 220-221.

Posi- tives with 10 ml.	Prob- able No. per 100 ml.	Positives with 10 ml. 1 ml.		Prob- able No. per 100 ml.	Positives with 10 ml. 1 ml. 0.1 ml.			Prob- able No. per 100 ml.	Positives with 10 ml. 1 ml. 0.1 ml.			Prob- able No. per 100 ml.
0	0	0	0	0	0	0	0	0	1	0	0	2
1	2.2	0	1	1.8	0	0	1	1.8	1	0	1	4
2	5.1	0	2	3.7	0	0	2	3.6	1	0	2	6
3	9.2	0	3	5.6	0	0	3	5.4	1	0	3	8
4	16	0	4	7.5	0	0	4	7.2	1	0	4	10
5	18+	0	5	9.6	0	0	5	9	1	0	5	12
		1	0	2.0	0	1	0	1.8	1	1	0	4
		1	1	4.1	0	1	1	3.6	1	1	1	6.1
		1	2	6.2	0	1	2	5.5	1	1	2	8.1
		1	3	8.4	0	1	3	7.3	1	1	3	10
		1	4	11	0	1	4	9.1	1	1	4	12
		1	5	13	0	1	5	11	1	1	5	14
		2	0	4.5	0	2	0	3.7	1	2	0	6.1
		2	1	6.9	0	2	1	5.5	1	2	1	8.2
		2	2	9.4	0	2	2	7.4	1	2	2	10
		2	3	12	0	2	3	9.2	1	2	3	12
		2	4	15	0	2	4	11	1	2	4	15
		2	5	17	0	2	5	13	1	2	5	17
		3	0	7.9	0	3	0	5.6	1	3	0	8.3
		3	1	11	0	3	1	7.4	1	3	1	10
		3	2	14	0	3	2	9.3	1	3	2	13
		3	3	18	0	3	3	11	1	3	3	15
		3	4	21	0	3	4	13	1	3	4	17
		3	5	25	0	3	5	15	1	3	5	19
		4	0	13	0	4	0	7.5	1	4	0	11
		4	1	17	0	4	1	9.4	1	4	1	13
		4	2	22	0	4	2	11	1	4	2	15
		4	3	28	0	4	3	13	1	4	3	17
		4	4	35	0	4	4	15	1	4	4	19
		4	5	43	0	4	5	17	1	4	5	22
		5	0	24	0	5	0	9.4	1	5	0	13
		5	1	35	0	5	1	11	1	5	1	15
		5	2	54	0	5	2	13	1	5	2	17
		5	3	92	0	5	3	15	1	5	3	19
		5	4	160	0	5	4	17	1	5	4	22
		5	5	180+	0	5	5	19	1	5	5	24

this case, the result would be $\frac{5}{6}$ instead of $\frac{5}{5}$. Thus the result

1 ml.

$\frac{5}{5}$

0.1 ml.

$\frac{5}{5}$

may be read

1 ml.

$\frac{5}{5}$

0.1 ml.

$\frac{5}{6}$

giving a probable number of 1,800.

In the table such a minimum probable number is given with the plus sign (+) appended, indicating that the probable number is at least that given, but may be greater.

TABLE OF MOST PROBABLE NUMBERS USING FIVE TUBES WITH EACH DILUTION¹⁶
 More complete tables will be found in the *Standard Methods of Water Analysis*, 8th Ed., 1936, pp. 220-221.

Positives with 10 1 0.1 ml. ml. ml.	Prob- able No. per 100 ml.	Positives with 10 1 0.1 ml. ml. ml.	Prob- able No. per 100 ml.	Positives with 10 1 0.1 ml. ml. ml.	Prob- able No. per 100 ml.	Positives with 10 1 0.1 ml. ml. ml.	Probable No. per 100 ml.
2 0 0	4.5	3 0 0	7.8	4 0 0	13	5 0 0	23
2 0 1	6.8	3 0 1	11	4 0 1	17	5 0 1	31
2 0 2	9.1	3 0 2	13	4 0 2	21	5 0 2	43
2 0 3	12	3 0 3	16	4 0 3	25	5 0 3	58
2 0 4	14	3 0 4	20	4 0 4	30	5 0 4	76
2 0 5	16	3 0 5	23	4 0 5	36	5 0 5	95
2 1 0	6.8	3 1 0	11	4 1 0	17	5 1 0	33
2 1 1	9.2	3 1 1	14	4 1 1	21	5 1 1	46
2 1 2	12	3 1 2	17	4 1 2	26	5 1 2	64
2 1 3	14	3 1 3	20	4 1 3	31	5 1 3	84
2 1 4	17	3 1 4	23	4 1 4	36	5 1 4	110
2 1 5	19	3 1 5	27	4 1 5	42	5 1 5	130
2 2 0	9.3	3 2 0	14	4 2 0	22	5 2 0	49
2 2 1	12	3 2 1	17	4 2 1	26	5 2 1	70
2 2 2	14	3 2 2	20	4 2 2	32	5 2 2	95
2 2 3	17	3 2 3	24	4 2 3	38	5 2 3	120
2 2 4	19	3 2 4	27	4 2 4	44	5 2 4	150
2 2 5	22	3 2 5	31	4 2 5	50	5 2 5	180
2 3 0	12	3 3 0	17	4 3 0	27	5 3 0	79
2 3 1	14	3 3 1	21	4 3 1	33	5 3 1	110
2 3 2	17	3 3 2	24	4 3 2	39	5 3 2	140
2 3 3	20	3 3 3	28	4 3 3	45	5 3 3	180
2 3 4	22	3 3 4	31	4 3 4	52	5 3 4	210
2 3 5	25	3 3 5	35	4 3 5	59	5 3 5	250
2 4 0	15	3 4 0	21	4 4 0	34	5 4 0	130
2 4 1	17	3 4 1	24	4 4 1	40	5 4 1	170
2 4 2	20	3 4 2	28	4 4 2	47	5 4 2	220
2 4 3	23	3 4 3	32	4 4 3	54	5 4 3	280
2 4 4	25	3 4 4	36	4 4 4	62	5 4 4	350
2 4 5	28	3 4 5	40	4 4 5	69	5 4 5	430
2 5 0	17	3 5 0	25	4 5 0	41	5 5 0	240
2 5 1	20	3 5 1	29	4 5 1	48	5 5 1	350
2 5 2	23	3 5 2	32	4 5 2	56	5 5 2	540
2 5 3	26	3 5 3	37	4 5 3	64	5 5 3	920
2 5 4	29	3 5 4	41	4 5 4	72	5 5 4	1600
2 5 5	32	3 5 5	45	4 5 5	81	5 5 5	1800+

NOTE: In the interest of uniformity this table, consisting of portions (rearranged and amplified) of the table presented in *Standard Methods of Water Analysis*, Eighth Edition, 1936, has been substituted for that of a previous (Seventh) edition of the *Standard Methods for the Examination of Dairy Products*.

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G. TESTS FOR PATHOGENIC, USUALLY HEMOLYTIC STREPTOCOCCI

Examine samples of milk for the presence of beta hemolytic streptococci under any one of the following circumstances: (a) Where milk supplies are thought to have caused septic sore throat or scarlet fever epidemics, (b) where routine examination of animals in herds producing raw milk are to be made, and (c) where pasteurized milk supplies are to be examined for the presence of streptococci associated with human diseases.

I. MILK SUPPLIES THOUGHT TO HAVE CAUSED SEPTIC SORE THROAT OR SCARLET FEVER EPIDEMICS

Examine all milk supplies thought to have caused such epidemics for beta hemolytic streptococci. Chief emphasis should be placed upon a quick detection of the cow discharging the organisms causing the epidemic and immediate elimination of any infected animals from the milking line.

Examine milk from quarters showing abnormalities in preference to milk from other quarters. It should not be concluded, however, that other cows may not be discharging the sought-for streptococci. Clinical manifestations cannot be relied upon to reveal cows infected with streptococci of the kind commonly regarded as pathogenic for man as almost identical macroscopic pathologic changes in the udder as well as the milk may be brought about by streptococci associated with bovine diseases. Conversely, udders that appear normal on superficial examination may harbor streptococci pathogenic to man.

1. METHODS OF SECURING SAMPLES

Care should be taken to secure samples as aseptically as possible where individual quarter samples are to be examined. Where a general milk supply is to be examined, it is advisable to examine not only a well mixed sample of the milk but also a sample of gravity cream.

If samples cannot be examined immediately, approximately 30 per cent glycerol¹ may be added to maintain the samples in good condi-

tion until they are examined. One per cent brilliant green added at the rate of 0.1 ml. per 10 ml. of milk (1:10,000 dilution) has also been recommended.²

2. APPARATUS AND MATERIALS

A. EQUIPMENT

Sterile sampling vials or tubes are required to secure samples from suspected quarters. These containers may be either glass stoppered bottles, screw top vials, rubber or cork stoppered test tubes, or any suitable type of container which can be sterilized and handled in the number required. Where large numbers of individual quarter samples are to be examined, cork stoppered test tubes are the most convenient. Care should be taken to insure sterility of the cork stoppers, particularly where stoppers are used more than once. Do not use cotton-stoppered test tubes or bottles.

B. MEDIA

Media for use in detecting hemolytic streptococci in milk are prepared as follows:

Veal (or Heart) Infusion Broth—Five hundred grams of finely ground veal (or heart muscle) are infused overnight in the icebox, in 1 liter of distilled water, boiled until infusion is clear and coagulum is brown, strained through cheese cloth and filtered through paper, 5 gm. each of sodium chloride and tryptone added, and the reaction adjusted to pH 7.2. The infusion is filtered and then sterilized by heating to 121° C. for 20 minutes.

Veal (or Heart) Infusion Agar—Veal (or heart) infusion broth prepared as above 1,000 ml. and agar 20 gm. Add 5 per cent defibrinated horse or rabbit (less is known about the appearances when beef blood is used) blood to the agar cooled at 45° C. just prior to pouring plates.

*Tryptone Blood Agar*³—Prepare 2.0 per cent agar (best quality, not oven dried) containing 0.5 per cent tryptone, and 0.5 per cent sodium chloride. Add 5 per cent of defibrinated horse blood to the agar, cooled to 45° C. just prior to pouring plates.

The agar may be stored in small flasks for plating, or in test tubes in 10 ml. amounts. If Burri agar slants are to be used, the agar should be tubed, slanted, and the slants allowed to dry in a horizontal position until no water of condensation is present.

3. METHODS OF ISOLATION

The following are suggested as routine preliminary or presumptive procedures to detect quickly the source of the infection of the suspected supply. The presence of definite beta hemolytic colonies should serve as a presumptive test for the recognition of infected animals. If desired, subcultures may be subjected to further identification by the methods outlined in the Report of the Committee on Diagnostic Procedures and Reagents, in order to establish the identity of any streptococci isolated.

Two procedures are available for the original isolation, viz., blood agar plates⁴ and Burri agar^{5, 6} slants. In the blood agar plate method, 10 ml. of the agar are melted and cooled to 45° C. and 0.5 ml. defibrinated blood is added immediately prior to pouring the agar.

The blood agar plate method is usually used although the Burri slant method is preferable where a large number of samples are to be examined in a routine way as the technic is less complicated and test tubes are used in place of Petri dishes. Quarters discharging streptococci may be quickly and easily found by the Burri method.

Plate Method—The preparation of agar plates and dilutions shall follow the same general procedure as that outlined for the standard agar plating procedure. Incubate plates at 37° C. for 48 hours and examine. Include uninoculated blood agar plate controls as a test of the sterility of the blood in all cases. Include as controls, test organisms that are known to produce beta hemolysis in order to demonstrate that the medium is satisfactory.

Burri Slant Method—For inoculation of the agar slants use a platinum loop 1.0 mm. in diameter made from wire 0.3 mm. in diameter. Flame the loop and withdraw a loopful of milk. Touch it to the surface of the agar slant (no blood added) in three places beginning at the bottom of the slant. Then streak the inoculum by drawing the loop in a zigzag way across the surface of the slant, beginning at the bottom and continuing to the top.

Definite colonies are usually visible on the slants after incubation at 37° C. for 48 hours (Figure XVIII). Examine preparations from colonies that have the appearance of streptococcus colonies by the Gram procedure. Test colonies found to be streptococci subsequently on blood agar plates and if the streptococci are found to be hemo-

lytic, reserve them for more complete identification. While this is a longer procedure than to plate directly on blood agar, only those samples that contain streptococci need to be plated and hemolytic streptococci have sometimes been found by this method where they have failed to appear on blood agar plates made directly from original samples.



FIGURE XVIII—Burri Slants Prepared from Freshly Drawn Milk.

A and B show a predominance of *Streptococcus* colonies; C and D show only *Micrococcus* colonies. In the latter case, the colonies may be either white, yellow, or orange, depending upon the type of *Micrococcus* present.

4. IDENTIFICATION OF THE SPECIES OF STREPTOCOCCI PRESENT

Various species of streptococci may be encountered in ordinary milk supplies. The so-called lactic acid streptococci (*Streptococcus lactis* Löhnis and *Streptococcus cremoris* Orla-Jensen) are rare or absent from samples drawn aseptically from the udder. These are generally inactive or green-producing (alpha or viridans) on blood agar. The lactic acid streptococci, however, may be abundant in market milk samples. Another common species of streptococcus is the streptococcus of bovine mastitis (*Streptococcus agalactiae* Lehmann and Neumann) which appears as a green-producing (viridans), a weakly hemolytic (alpha prime or narrow zoned)

colony,^{7, 8} or even with a broad zone of hemolysis on blood agar plates. This species may be encountered in all kinds of raw milk samples and can be readily differentiated by cultural and serological tests from other species of streptococci.

The most significant species from the public health standpoint are those of Lancefield's group A, *Streptococcus pyogenes* Rosenbach (may be referred to by some as *Streptococcus hemolyticus* Rolly or certain varieties may be called *Streptococcus epidemicus* Davis, *Streptococcus scarlatinae* Klein, or by other names). The variety of this species associated with septic sore throat epidemics is commonly, although not invariably, capsulated.

5. IDENTIFICATION OF THE STREPTOCOCCI WHICH PRODUCE BETA HEMOLYTIC COLONIES

Presumptive Test—Examine the blood agar plates for colonies which have the following characters: Typical beta colonies with a wide zone of hemolysis which develops after 18 to 24 hours of incubation. The deep colonies of suspected human origin are dense and are surrounded by a wide clear zone with a small colony. Make Gram stains from deep colonies and broth cultures to determine whether the suspected organism is a streptococcus. The finding of a Gram-positive, beta hemolytic streptococcus in a freshly drawn quarter sample should be interpreted as a presumptive positive finding and is sufficient to warrant the elimination of the cow from the herd.

Confirmatory Tests—Confirm the identity of the streptococcus where desired by determining its cultural characters and the serological group to which it belongs. Directions for carrying out these tests will be found in the report on Diagnostic Procedures and Reagents published by the American Public Health Association (First Edition, 1941).

It should be remembered that before a relationship between a milk supply and an epidemic can be regarded as established, it should be shown that the strains from throat cultures secured from patients and the strains secured from the suspected milk give identical reactions.

6. IDENTIFICATION OF THE BOVINE MASTITIS STREPTOCOCCUS

It has been found that a large percentage of udders discharge streptococci.⁹ These streptococci are usually of the species associated

with latent, subclinical, or active mastitis, and are not believed to have public health significance. These bovine forms rarely produce broad zones of hemolysis around the colony on blood agar plates. Frequently the hemolysis is of the alpha (viridans) type. In some instances on aerobic plates narrow zones of clear hemolysis may develop, or by alternating incubation with refrigeration concentric zones of hemolysis may occur known as double zone hemolysis.

II. ROUTINE EXAMINATION OF ANIMALS IN HERDS

Routine control of herd milk is best accomplished by a regular routine examination of samples from individual quarters of the udder. If examination of individual quarter samples involves too great effort, it is advisable to plate group samples from not more than ten cows for the presence of beta hemolytic streptococci. If found to be present, the individual cows in the suspected group should be examined and the offending individual eliminated from the herd.

It should be noted that the failure to find infected animals by routine examinations does not safeguard the milk supply perfectly. Infected animals may appear in the intervals between examinations or they may be overlooked.

III. PASTEURIZED MILK

Beta hemolytic streptococci are frequently found in pasteurized milk. Little is known in regard to their origin and significance. There is no evidence to indicate that they are of public health importance, and there is much reason for regarding them as harmless saprophytes. It is generally recognized that the species of beta hemolytic streptococci (*Streptococcus pyogenes*) associated with human diseases does not survive pasteurization temperatures.

If pasteurized milk is to be examined, it should be plated as outlined above and the beta hemolytic colonies, if present, isolated and studied. The species usually found in pasteurized milk are known as *Streptococcus durans* Sherman, or as *Streptococcus zymogenes* Holland.^{10, 11} Neither of these is regarded as pathogenic for man.

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H. EXAMINATION OF MILK FOR TUBERCLE BACILLI

I. TYPES OF TUBERCLE BACILLI IN MILK

Tubercle bacilli in milk are nearly always of the *bovine* type. Bacilli of the *human* type may be found in milk but only when it has been grossly contaminated with the discharges, especially sputum, of a tuberculous milker or handler. Bacilli of the *avian* type may possibly get into milk occasionally through contamination with fecal material or uterine discharges and consequently in small numbers. Since this type presents little or no danger to man or the domestic mammals other than swine, it is regarded as of such slight consequence that it will not be discussed further here.

None of the tubercle bacilli multiply in milk; hence large numbers always mean gross contamination of the supply. Under natural conditions only bacilli of the *bovine* type can be present in large numbers and this because of the discharge of tuberculous pus into the milk from lesions in the cow's udder. Fortunately, tuberculosis localizes in the udder of cows in only a small percentage of the cases, so that large numbers of bacilli are found in market milk only rarely. In areas where bovine tuberculosis is common, the mixed milk of dairies very commonly contains living tubercle bacilli, which, even though they may not be present in large numbers, constitute an important public health matter especially for children and adolescents. In the United States this hazard has practically been eliminated through the Accredited Herd Movement which has reduced the incidence of bovine tuberculosis to a very low level and has succeeded in eliminating in large measure the more advanced cases which are the principal spreaders of infection. It has been claimed that cattle may excrete tubercle bacilli in the milk when disease of the udder is not present, the organisms in these cases escaping by way of the blood from tuberculous abscesses of the lungs or other organs where the disease more commonly localizes. If this occurs the numbers of bacilli that reach the milk must be small. Considerable numbers of bovine tubercle bacilli may reach milk through contamination with fresh or partly dried fecal material from the flanks and udder of the cow, since the feces of cows suffering from advanced pulmonary disease often are rich in bacilli

because of the presence of sputum which is swallowed after being coughed up. Cows sometimes suffer from tuberculosis of the uterus, in which cases the discharge may be very rich in bacilli. This discharge may easily contaminate the milk if the milking methods are not of the best.

II. CONCENTRATION OF TUBERCLE BACILLI IN MILK

Because milk usually contains relatively few tubercle bacilli it is desirable, no matter what method of examination is being used, to concentrate the organisms present. This is usually done by centrifuging or by allowing the milk to stand for 24 hours or longer in a refrigerator. By either method the fat or cream rises to the surface and most foreign substances, such as bits of dirt, manure, fragments of tissue that may have originated in tuberculous ulcers, and leucocytes, settle to the bottom. Most of the bacteria present, tubercle bacilli among them, will be found in these two layers. The examination, therefore, should be made of these two portions, either separately, or by mixing them together and examining the mixture.

If large quantities of milk are available, pint or quart samples are placed in sterile bottles or cylinders which are allowed to stand in the refrigerator for 24 hours. The cream and sediment layers are collected by siphoning. These are mixed and centrifuged in 250 ml. sterilized bottles at high speed for 30 minutes. The top and bottom layers are collected, mixed together, and the mixture used for animal inoculation.

III. METHODS OF EXAMINATION

There are three methods of examining milk for tubercle bacilli. These are: (1) the direct microscopic method, (2) cultural methods, and (3) the animal inoculation method. These will be discussed in turn.

1. DIRECT MICROSCOPIC EXAMINATION

Because of their acid-fastness tubercle bacilli may be readily recognized in films prepared from milk sediment. When present in small numbers they may not be found, and when found some caution should be observed in positively identifying them as tubercle bacilli. It is probable that not many errors would be made, yet many saprophytic acid-fast organisms exist, some of which can be distinguished from tubercle bacilli on a morphological and tinctorial

basis with difficulty or not at all and these may find their way into milk. One may encounter, especially when dealing with cream, artifacts that may be interpreted as acid-fast bacilli. Because of the collection of fatty substances around ordinary bacteria or other small objects, decolorization of these bodies may be prevented, and they thus assume the appearance of acid-fastness. It is good practice to rinse the fixed films, before staining, with a good fat solvent such as ether or xylol to prevent these effects.

Probably as reliable a method as any for staining acid-fast organisms is the Ziehl-Neelson method. After staining with steaming carbol-fuchsin solution for at least 3 minutes, the films are decolorized for at least 10 seconds in 95 per cent alcohol containing 3 per cent of concentrated hydrochloric acid. Counter staining for about 10 seconds in Loeffler's methylene blue solution is advised.

2. CULTURAL METHODS

Tubercle bacilli may be cultivated directly from milk after treating it with an agent capable of destroying the non-acid-fast organisms present. Wolters¹ recommends the use of hydrochloric acid followed by cultivation on an egg medium containing malachite green. Quite reliable results can be obtained by an experienced person with this, and other cultural methods and they are described in the report on Diagnostic Procedures and Reagents published by the American Public Health Association (First Edition 1941). Acid-fast organisms which appear in cultures should not be regarded as tubercle bacilli without confirmatory evidence. The average worker will succeed better with the animal inoculation method next to be described.

3. ANIMAL INOCULATION METHOD

Guinea pigs are highly susceptible to infection with mammalian tubercle bacilli and are the best experimental animals for detecting tubercle bacilli in milk. They are not susceptible to avian bacilli, however; hence, if this type is sought, other animals, such as rabbits or chickens, should be used.

Animals weighing at least 350 gm. are preferred to smaller animals since the former are less apt to succumb to the effects of extraneous organisms that may be in the milk. If the history of the animals is unknown or there is any reason to suspect that they may be infected, test them with tuberculin intradermally.

Inoculate intraperitoneally using quantities up to 5 ml. The quantity injected should depend on the quality of the milk and its age. Dirty milk and old milk with a high bacteria count naturally are more likely to cause death from peritonitis than better grades, and smaller quantities should be injected. At least two animals should be injected with each sample. It is well to retain in the refrigerator a part of each sample so that if all the animals inoculated with some of them die within a day or two, others may be inoculated with smaller quantities.

If the milk samples have been collected especially for examination for tubercle bacilli, and particularly if they must be shipped, it is well to draw them into bottles containing enough boric acid to make a 1 per cent solution in the milk, as recommended by Traum and Hart.² The boric acid does not harm the tubercle bacilli but will prevent multiplication of other organisms.

If possible the inoculated guinea pigs should be kept in small cages with water-tight bottoms, and only those pigs that have been inoculated with the same sample allowed to associate with each other.

All animals that die should be carefully autopsied soon after death. Caseous masses in the great omentum should be looked for; also the characteristic lesions of the liver, spleen, and lungs. Tubercle bacilli are not always easily found in smears of these lesions. In case the nature of the lesions is not entirely clear, it is best to inoculate other guinea pigs with them. In this case the placing of the inoculum in the muscles of the thigh rather than in the peritoneal cavity has the advantage that one can trace the course of the infection through the lymphatics until it reaches the visceral organs. An experienced worker rarely has any difficulty in deciding whether or not lesions of guinea pigs are due to tubercle bacilli even though the acid-fast organisms are not demonstrated. In exceptional cases, where doubt exists, one may resort to cultivation of the bacillus, a procedure which offers no difficulty providing suitable media are used.

After about 6 weeks many workers like to subject the test animals to a tuberculin test. This may be done by injecting intradermally into a shaved or plucked area of the abdomen about 0.1 ml. of the regular concentrated tuberculin used for testing cattle. The tests are read in 48 hours. For details of the method see Hagan and Traum.⁴ Another method is to inject intraperitoneally the equivalent

or about 0.5 ml. of O.T. (old tuberculin). Tuberculous guinea pigs are usually killed by such a dose within 12 to 24 hours. These animals, of course, are subjected to the regular autopsy examination.

Surviving animals are destroyed not sooner than 8 weeks after inoculation and 12 weeks is preferred if time is not pressing.

Usually when one animal of a pair is found to be tuberculous the other will also show the disease. Sometimes, however, only one of a group of animals will exhibit the disease. This happens particularly with milk that has been underpasteurized and no doubt indicates that very few viable bacilli are present.

Infected animals usually show progressive disease with extension of the lesions to several of the internal organs. Occasionally, however, and especially when dealing with milk that has been heated, only a localized disease will be found. These animals should be very carefully examined and, if the evidence is not conclusive that the disease is tuberculosis, reinoculations should be done, or the bacillus should be cultivated.

The fact should always be kept in mind that lesions simulating those of tuberculosis can be produced in experimental animals by the injection of any acid-fast organism. The saprophytic acid-fast organisms must be introduced in comparatively large numbers to cause such an effect, however, and probably it seldom happens that milk contains enough to do it. When they are suspended in a fatty material, such as cream, the power of such organisms to produce lesions is greatly increased, as was first pointed out by Rabinowitsch,³ and recently studied in more detail by Hagan and Levine.⁵ If it is suspected that lesions are pseudo-tuberculous, it is an easy matter to settle the question by injecting other guinea pigs. The true tuberculous infection will, of course, appear in them, whereas the pseudo-disease will not. Furthermore, the saprophytic acid-fast organisms grow easily and rapidly on culture media and may be identified in this way.

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I. PROCEDURES FOR THE DETECTION OF BRUCELLA SPP. IN MILK

During the past 10 years sufficient evidence has been accumulated by many investigators in both Europe and North America to prove beyond question that the two species, *Brucella abortus* and *Brucella suis*, as well as *Brucella melitensis*, are pathogenic for human beings. The symptom-complex resulting from infection with any of the three species has thus far defied differentiation.

Infection in human beings may be acquired by way of the cutaneous system through the handling of infected animals and their excretions, or by way of the mucosa through the ingestion of dairy products containing the living organism.

The following species of animals have been found naturally infected with *Brucella*: cows, goats, sheep, horses, dogs, wild deer, wild buffalo, hogs and fowls. All three species of *Brucella* have been isolated from infective cow's milk.

I. DESCRIPTION OF THE SPECIES 1, 2, 15

For primary isolation on culture media, the inoculated cultures must be incubated for 5 days at 37° C. in an atmosphere containing approximately 10 per cent carbon dioxide. Subsequent transfers for about 15 times require the same amount of carbon dioxide for growth. If infective material is cultured from an animal inoculated with a strain which has become aerobic through subsequent transfers, an increased carbon dioxide atmosphere is not necessary for primary isolation.

The colonial appearance of the smooth form of the three species is about the same. The colony is spheroidal in shape, slightly bluish in color, and translucent. The size varies from 3 to 5 mm. diameter. Colonies on direct culture from milk occasionally may be flattened. These are large and are about 7 mm. in diameter. When viewed from the top, the growth on the surface of slants has a moist, greasy appearance. The surface growth of *Brucella melitensis* may become brown with age, the brownish coloration oftentimes extending down into the medium. Rough colonies of the species of *Brucella* are grey in color and opaque. They may be identified and distinguished from smooth colonies by following the technic described by Henry.³

The smooth forms of *Brucella* possess a capsule which may be demonstrated by the use of a suitable technic.² The rough forms are without a capsule.

The optimum pH for the growth of the three species on solid or liquid media is 6.6 or 6.8.

Detailed description of the three species of *Brucella* commonly found in milk will be found in standard books.^{1, 15}

II. THE ISOLATION OF BRUCELLA SPP. FROM MILK^{3, 5, 15}

1. GUINEA PIG INOCULATION METHOD

If udders of an individual animal are to be examined⁴ for the presence of bacteria belonging to the genus *Brucella* the milk should be collected at or near milking time.

Wipe the teats with a clean damp cloth and discard the first two or three streams of milk from each quarter. Take samples of milk for examination from the hind quarters in one sterile test tube and from the front quarters in another sterile test tube. Take about 15 ml. of milk in each tube, or 7.5 ml. from each quarter. Allow the tubes of milk to stand in a cold room for 24 hours in order to permit the cream to rise to the surface. The cream carries with it the majority of the organisms in the milk. Inject the entire cream layer of each sample intraperitoneally or subcutaneously into a healthy guinea pig. Place inoculated guinea pigs in individual cages and kill at the end of 6 weeks. Examine the tissues of the pigs for lesions characteristically produced by bacteria of the *Brucella* group. Make cultures from suspected lesions. Test the blood serum of the pigs in order to detect the presence of *Brucella* agglutinins.

If bottled market milk is to be examined, the entire cream layer should be removed from the top of the milk, placed in a sterile tube or flask, and mixed thoroughly. Inject guinea pigs either subcutaneously or intraperitoneally with 2 ml. of the cream.

If a sample of milk is to be shipped to the laboratory from a considerable distance for examination and two or three days may pass before it arrives, 1 per cent boric acid should be added to the milk as recommended by Traum and Henry.⁶ The addition of crystal violet to the milk will serve the same purpose as boric acid. The final dilution of the dye should be 1:25,000.

2. PLATE METHOD

This method offers a rapid, accurate, and quantitative means of detecting the presence of bacteria of the genus *Brucella* in milk drawn directly from the udder of the animal.⁷ Collect the milk samples in the same manner and with the same (precautions) as for guinea pig inoculation. After the milk samples have stood in a cold room for 24 hours, draw off the cream with a pipette and culture by placing 0.1 to 0.2 ml. on the surface of each of two liver agar plates (0.5 per cent Bacto peptone) or tryptose agar (Difco)* plates containing crystal violet in a final dilution of 1:700,000. The crystal violet may be prepared in a 0.1 per cent aqueous stock solution. The dye inhibits the growth of many Gram-positive organisms commonly found in milk. Spread the drops of cream over the surface of the plate by means of a sterile glass rod with a right-angle bend, by rotating the plate in a horizontal plane. The plates are placed in a container) such as is shown in Figure XIX. The container is closed and 10 per cent of the air is replaced with carbon dioxide. The inoculated plates are incubated in the container for 5 days at 37° C. and examined for colonies of *Brucella spp.* When present, they have a clear light blue violet appearance on liver agar containing crystal violet. The size of the colonies varies from 2 to 7 mm. in diameter. The total number of colonies which appear on a plate varies from 5 to 10,000 and depends upon the degree of infection in the quarter of the udder. Suspicious colonies should be transferred to agar slants or to another crystal violet liver agar plate if the inoculated plate contains colonies of other bacteria or molds. The suspicious cultures should be identified as *Brucella* by means of the agglutination test using an agglutinating serum of known titer and also a normal serum.

If aerobic types of *Brucella*, such as *Brucella suis* or *Brucella melitensis*, are present in the milk, their growth will not be inhibited by incubating the inoculated plates in an atmosphere of 10 per cent carbon dioxide. If the first transfer of a primary culture grows aerobically, it is presumptive evidence that the species is not *Brucella abortus*. Since it has been found that strains of *Brucella abortus* which have become aerobic through artificial cultivation may be recovered aerobically from animals following inoculation, it is evident that one must depend upon other methods to determine to which species a newly isolated aerobic strain of *Brucella* belongs.

* Bacto-tryptose 20 gm., Bacto-dextrose 1 gm., sodium chloride 5 gm., Bacto-agar, 20 gm.

The direct isolation of bacteria belonging to the genus *Brucella* from market milk is difficult if streptococci are also present in the cream. Streptococci, although Gram-positive, are not inhibited in their growth by the gentian violet solutions of the strength specified

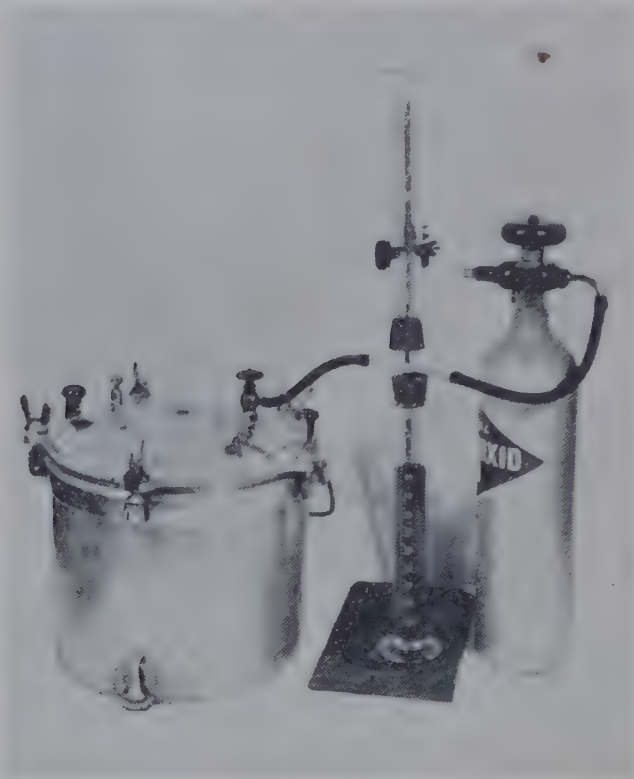


FIGURE XIX—Apparatus for obtaining CO_2 and culture container for the isolation of *Brucella abortus*. Apparatus shown consists of an ordinary pressure cooker with the pressure gauge removed, a CO_2 tank, and two graduated cylinders one of which is inverted over the other. A one-way metal stop-cock is inserted in the pressure gauge opening for removing air by means of a vacuum pump and receiving the CO_2 collected and measured over a column of water in the graduated cylinder. The two cylinders are connected together by means of glass tubing. The gas coming from the tank is received in the lower cylinder, forcing the water upward into the inverted cylinder. When the required amount of gas is collected in the lower cylinder, the flow of gas from the tank is closed. The cock on the pressure cooker is opened allowing the collected gas to flow into the cooker. There are three sizes of the cookers on the market. The average laboratory will need only the smaller one.

above. Streptococci appear to change the pH of the medium to such a degree that bacteria belonging to the genus *Brucella*, if present in the same milk or cream, will not appear as visible colonies.

In isolating *Brucella melitensis* from the milk of the goat or sheep one may follow either of the two methods described for detecting *Brucella abortus* in cow's milk.

III. EXAMINATION OF MILK (COW, GOAT AND SHEEP) FOR BRUCELLA AGGLUTININS 8, 9, 15

1. COLLECTION AND PREPARATION OF MILK SAMPLES

Milk samples are collected, preferably from individual quarters of the udder, in clean test tubes or vials. The strippings should never be used. The tubes should be filled to only one-half their volume. Let the samples stand from 6 to 8 hours to permit the cream to separate. Remove as much of the cream as possible with a pipette. Add rennet (1 per cent solution), allowing 2 drops for each 5 ml. of milk, to each tube of milk, and mix thoroughly. The tubes may be placed either in an incubator at 37° C. or in warm water at the same temperature. If the tubes are placed in a slanting position, the curd will settle to one side, and the clear milk serum with which the test is to be made will separate in about 2 hours. In order to obtain a serum free from particles of casein, the samples should be placed in an icebox or cold place for from 6 to 8 hours before testing. Sour and decomposed milk should not be used, as the results are not reliable. Neither should milk be used in which the curd has become partly digested as this interferes with the test. Colostrum is unsatisfactory because of the difficulty of separating the serum and the possible occurrence of nonspecific agglutinins.

2. THE AGGLUTINATION TEST

One may choose either of two methods, namely the test tube or rapid agglutination test.

A. TEST TUBE METHOD

A smooth strain of *Brucella abortus* is grown on liver agar slants for 48 to 72 hours at 37° C. The growth is removed and suspended in physiological salt solution containing 0.5 per cent phenol. The final turbidity of the suspension should measure 7 cm. by the Gates wire loop method or Tube I by the McFarland method.¹⁵ Place 2 ml. of the antigen into a series of 5 agglutination tubes. To the first tube add 0.08 ml. of milk serum, the second 0.04 ml., the third 0.02 ml., the fourth 0.01 ml., and to the fifth 0.005 ml. These amounts of milk serum added to 2 ml. of antigen will give approximate dilutions of 1:25, 1:50, 1:100, 1:200, and 1:400. No

further dilutions are necessary in making routine examination of milk for *Brucella* agglutinins.

Serum and antigen are mixed thoroughly in the tubes and incubated for 48 hours at 37° C.

The following system may be used in recording changes in the stability of the antigen: + = complete sedimentation; P = incomplete sedimentation; — = no sedimentation. The presence of infection in the udder is indicated by complete agglutination in a 1:50 dilution or above of serum.

B. RAPID AGGLUTINATION TEST ¹⁵

The rapid agglutination test offers a simple and accurate method of detecting *Brucella* agglutinins in milk from any species of animal infected with any one of the three species of *Brucella*.

Materials Needed—(1) darkfield illumination box and glass plate ruled into squares; (2) 0.2 ml. pipettes graduated in 0.01 ml.; (3) clean toothpicks for mixing milk serum and antigen; (4) properly standardized antigen and standardized dropper pipette.

Technic—In beginning the test, arrange the serum samples in a row parallel with the darkfield illumination box. The glass plate, with the etched squares upward, is placed over the opening of the box and the identification number of the serum sample marked with a wax pencil on either the top or bottom of the row of squares used.

With a clean 0.2 ml. pipette, draw up serum from the first milk sample to the zero mark on the pipette. Beginning in the bottom left hand square of the plate, place the following amounts of serum in the succeeding squares toward the top; 1st square, 0.08 ml.; 2nd square, 0.04 ml.; 3rd square, 0.02 ml.; 4th square, 0.01 ml.; 5th square, 0.005 ml.

This manner of placing the serum brings the smallest amount farthest from the heat of the electric bulb in the box, reducing the rapidity of drying of the smallest amounts of serum. The procedure is continued, using the next set of vertical squares and a separate pipette for each sample. The best results are obtained by testing only 4 or 5 samples at a time as otherwise the small amounts of serum dry out too much before the test is completed.

If the pipette has been placed deep in the serum, there will be some serum which will collect on the outside at the tip. For accuracy, this should be removed by touching the tip of the pipette against the lip of the vial.

After thoroughly shaking the bottle of antigen, remove a dropper full of the antigen. Holding the dropper in a vertical position, add one drop to each amount of serum on the plate. Care should be taken to hold the dropper in a vertical position since holding it at another angle will make a considerable difference in the amount of antigen delivered. Always replace the dropper directly in the vial of antigen.

Then with a clean toothpick mix the serum and antigen, using a new toothpick for each sample. Always start at the top of the plate in the square containing the smallest amount (0.005 ml.) of serum and continue downward to the largest amount. Spread the mixture over about three-fourths the area of the square without coming in contact with the etched dividing lines.

Immediately after the samples have been mixed, remove the plate from the box and tilt slightly backward and forward slowly for about 5 minutes. Place the plate on the box, turn on the light, and record the results.

The reactions stand out very clearly. It is not difficult to distinguish between complete clumping of the antigen and different degrees of incomplete clumping. A negative serum causes no flocculation of the antigen. There are often encountered, however, sera which produce a trace of flocculation in the 0.08 ml. amount.

Immediately after using, the pipettes should be rinsed several times with fresh water until thoroughly clean. Then boil in distilled water and drain all the water out before using again. The glass plate may be cleaned with cleaning powder and brush, after which it is rinsed with distilled water and dried. By having several clean plates available, one can proceed with the testing of additional samples without delay. Absolute cleanliness of glassware is essential.

Interpretation of the Test—Rapid antigen has been standardized for use with undiluted serum in the following amounts: 0.08 ml., 0.04 ml., 0.02 ml., 0.01 ml., and 0.005 ml.

In the test tube method, when these amounts of serum are added to 2 ml. of antigen (turbidity 7 cm. by Gates apparatus), the following respective approximate dilutions are obtained: 1:25, 1:50, 1:100, 1:200, and 1:400.

The antigen has been standardized so that the titer of the agglutinins in a given serum will be parallel in the two methods. There-

fore, in the rapid test, complete agglutination with the various amounts of serum represents the following titers: 0.08 ml., 1:25; 0.04 ml., 1:50; 0.02 ml., 1:100; 0.01 ml., 1:200; 0.005 ml., 1:400.

If one desires to employ quantities of serum different from those just mentioned it will be necessary to standardize the antigen for the amounts in question. It is important to acquire an accurate knowledge of reading the test. If possible, a visit should be made to a laboratory conducting the test to obtain assistance in reading and interpreting the different degrees of agglutination.

The presence of infection in the udder is indicated by complete flocculence of the antigen with 0.04 ml. of milk serum. In the case of a composite milk sample, infection is indicated when flocculence occurs with 0.08 ml. of serum.

3. DIFFERENTIATION OF THE SPECIES OF *BRUCELLA*

It is important that one be thoroughly familiar with the peculiar growth characteristics of the *Brucella* group and the methods that have been successfully used in their differentiation, before an attempt is made to identify the species of a newly isolated strain. It is advisable for those laboratories which isolate cultures at wide intervals to forward new cultures for their identification to laboratory workers who have had considerable experience with the *Brucella* group.

There are five methods that have been used successfully with certain limitations, for the identification of the species of *Brucella*. The agglutinin-absorption method was used first by Feusier and Meyer¹⁰ for this purpose. It was studied more exhaustively by Evans¹¹ and by Wilson and Miles.¹² It is a valuable method for distinguishing *Brucella abortus* from *Brucella melitensis* if the technique employed by Wilson and Miles is followed, otherwise it will fail to distinguish one from the other in certain instances. The agglutinin-absorption method is not a reliable one for distinguishing rough strains of *Brucella melitensis* from rough strains of *Brucella abortus* nor will it separate *Brucella suis* from *Brucella abortus*.

The glucose utilization method, first applied and recommended by McAlpine and Slanetz¹³ for identifying the species of *Brucella*, has not found general acceptance by many other workers.

The difference in the nitrate and nitrite reducing ability of the species of *Brucella* has been made use of by Zobell and Meyer¹⁴ as a means of distinguishing one from the other. The results

obtained by this method have been confirmed by Huddleson.¹⁵ The method should be employed, wherever possible, in determining the species of a newly isolated culture.

The difference in the hydrogen sulfide metabolism of the three species of *Brucella* has been made use of by Huddleson¹⁵ in distinguishing one species from the other regardless of their source or whether the culture is smooth or rough. There is one group of strains which cannot easily be identified by the H_2S metabolism method. They are the Danish strains of *Brucella suis*. These strains do not produce H_2S to the same degree as other strains of *Brucella suis*.

A method¹⁵ which makes use of the difference in growth behavior of the species of *Brucella* toward certain aniline dyes in a solid medium has been generally accepted as the most satisfactory for identifying the species to which a given culture belongs.

The dye plate method should be followed in identifying the species of *Brucella*. The most suitable medium now available for differentiation of the species of *Brucella* is tryptose agar (Difco). This is a dehydrated complete medium. It is added to distilled water and sterilized according to the directions of the manufacturer. In its present form, it does not reduce the added aniline dyes as is often the case when liver agar is used. Reduction of the dyes permits growth of all three species and confuses differentiation.

The dyes which have been found to give consistent results in the differentiation of the three species are thionin and basic fuchsin. Use the certified dyes made by the National Aniline Division of the Allied Chemical Company.

The dyes are prepared in a 0.1 per cent stock solution or suspension in sterile distilled water. They are not readily soluble in water in this concentration. The stock solution of basic fuchsin does not keep well due to crystallization. It should be discarded after 10 days. The final dilution which should obtain for thionin and basic fuchsin in the medium is 1:100,000. These dilutions are based on the actual amount of the original dye in the medium.

The dye suspensions should be heated in flowing steam for 20 minutes, shaken well and, while still hot, added to the melted medium before it has time to cool. This procedure results in a more uniform mixture of the dye suspension and a more uniform distribution of the dyes in the medium. The medium and dyes are thoroughly

mixed and immediately poured into Petri plates. The plates are placed in a 37° C. incubator until the water of condensation disappears.

The plates may be divided into three or more sections to accommodate the growth of several strains of the organism. The seeding of the plates is accomplished with a loop of a heavy suspension of a 48 to 72 hour agar slant growth. The suspension may be obtained by working up a portion of the growth in the water of condensation at the butt of the slant or by adding a small amount of sterile broth or sterile saline solution to the slant. It may be stated here that the dyes do not kill the organism, but merely inhibit its reproductive function; so if masses of the culture are streaked over the surface of the plates, slight growth is very apt to occur at those points where the seeded mass has not been evenly distributed.

The seeded plates are incubated aerobically at 37° C. for 72 hours, or in 10 per cent CO₂ when newly isolated bovine strains are used. At the end of the period of incubation one will find that strains of the *Brucella abortus* species have grown only on the medium containing basic fuchsin; those of the *Brucella suis* species have grown only on the medium containing thionin; and those of the *Brucella melitensis* species have grown on each of the media. The growth of the last species, however, is as a rule never as luxuriant as that of the other two species.

IV. PREPARATION OF LIVER AGAR MEDIUM ¹³

Prepare the medium as follows: Grind fresh beef liver that is free from fat in a meat chopper until it forms a plastic mass. Place 1 pound of the liver and 500 ml. of distilled water in a covered container and mix well. Place the mixture in a cold room and allow to infuse for 24 hours. Remove the container and place in flowing steam for 20 minutes, removing the lid and stirring with a glass rod in order to heat all parts of the mixture. The heating is then continued in flowing steam for 1½ hours. Remove and filter through a wire screening. The infusion thus prepared is made either into an agar medium or is used as an infusion broth.

To prepare one liter of liver infusion agar, measure out the following ingredients:

Washed agar	20 gm.
Distilled water	500 ml.
Liver infusion	500 ml.

Peptone (Bacto)	5 gm.
Sodium chloride C.P.	5 gm.

Place all ingredients in a suitable container, cover and place in flowing steam for 1 hour. Remove and cool to 60° C. Adjust the pH at this time to 7.0. Place in flowing steam again for ½ hour. Decant and place in sterile flasks or tubes and sterilize at 15 lb. pressure for 30 minutes. The reaction of the medium during the process of sterilizing will usually drop to or near pH 6.6. The organisms grow best at or near pH 6.6. The final medium prepared according to the foregoing method will not be clear, but this will not interfere with its use as a medium. The final product may be freed from sediment and suspended particles if it is passed through a Sharples centrifuge before sterilization.

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J. MICROBIOLOGICAL METHODS FOR THE EXAMINATION OF BUTTER

Increasing attention is being given by food officials and the dairy industry to the microbiological content of butter. Butter differs from most other dairy products in that cultures of lactic organisms are frequently added to cream. These materially influence the total bacterial content of the finished butter. Yeasts and molds have accordingly been suggested as test organisms because they should be present only in very small numbers, if at all, in butter that is made from properly pasteurized cream, and then handled in thoroughly cleansed and commercially sterilized equipment. There are microbiological methods other than those used for detecting yeasts and molds which are used by the industry for determining butter quality, but none have met with such general acceptance as the various methods used for estimating the amount of yeast and mold growth.

The technics used for examining butter may be used for the determination of yeasts and molds in cream that is to be made into butter, for cultured butter milk, and for butter cultures used in the manufacture of butter.

I. METHOD OF SAMPLING

1. COLLECTION

Butter in the Churn—After the butter has been worked and is ready to be removed from the churn, take samples for microbiological analysis by means of a sterile trier, taking three samples of about $\frac{1}{3}$ oz. each, one from the center of the churn, and two from the respective ends.* Place the butter samples in sterile jars with screw or glass tops.

Tubs or Packages—Remove a total of at least 1 oz. of butter from

* Under commercial conditions where large numbers of samples must be taken daily, it may be impractical to employ a sterile trier for each sample. If a polished trier is wiped thoroughly after each sample with clean tissue paper until the surface is highly polished, dipped in ethyl alcohol, and the adhering alcohol burned off the trier, and then the trier plunged into the butter to be sampled at least twice before the sample is taken, satisfactory results may be obtained with a single trier.

two different parts of the tub or package with a sterile trier, the plugs of butter being not less than 2 in. in length and including the surface portion. Transfer the butter from the trier to the sterile sample jar with a sterile spatula or spoon.

Print Butter—Because of difficulty in obtaining the same amount of exposed surface of butter of 1, $\frac{1}{2}$, and $\frac{1}{4}$ lb prints, sample print butter by means of a trier. By using a small trier and plugging the end of the print, a plug of 3 to 4 in. may be obtained. Such a plug should weigh at least $\frac{1}{2}$ oz. Transfer the butter from the trier to a sterile sample jar with a sterile spatula or spoon.

2. CARE OF SAMPLES

Place all samples, immediately after sampling, in cracked ice, or place them in a refrigerator where the temperature does not exceed 40° F. (4° C.). Plate as soon as possible.

Store samples which are procured from stations remote from the laboratory at 4° C., or below until shipped. At no time prior to analysis should samples of butter containing less than 10 per cent salt in the serum be permitted to remain warmer than 4° C., nor samples of butter containing over 10 per cent to remain warmer than 60° F. (15.6° C.).

Cool and pack the samples for shipping carefully so as to prevent breakage and retain a low temperature. Upon receipt of the samples at the laboratory plate the samples as soon as possible. Record the period of time elapsed between sampling and plating.

II. TREATMENT OF EQUIPMENT

Sterilize glassware and equipment as specified in Section A, I, p. 7. Sterilize dilution bottles, media, etc., in the autoclave at 15 lbs. (121° C.) for at least 20 minutes after the pressure reaches 15 lbs.

Wrap metal triers, spatulas, and spoons either in paper (Kraft wrapping paper usually withstands sterilization temperatures without charring), or enclose them in metal or glass containers, and sterilize in the same manner as glassware.

Wooden tongue depressors make satisfactory spatulas. Wrap them in paper or enclose them in a metal or glass container, and sterilize by autoclaving or by hot air.

Use equipment such as pipettes, dilution bottles and Petri dishes, and water for dilution blanks, that agree with the specifications given in Section B, II, pp. 16-22.

III. PLATING PROCEDURE FOR DETERMINATION OF YEASTS AND MOLDS

1. PREPARATION OF SAMPLES FOR PLATING

Place the sample jar containing the butter to be tested in a water bath between 40 and 45° C., and rotate until the butter is of thin creamy consistency. Do not allow the time required for the sample to reach a creamy consistency to exceed 15 minutes. Pour the agar in the plates within 15 minutes from the time the plating procedure is started.

2. PREPARATION OF DILUTIONS

Wet and warm the 11 ml. pipettes to be used, by drawing 11 ml. of warm (40–45° C.) dilution water into the pipette; discharge, and then draw 11 ml. of thoroughly mixed melted butter into the pipette and discharge it into 99 ml. of sterile water. Care must be taken to keep the amount of butter adhering to the outside of the pipette at a minimum. Shake as in Section B, IV, 3, p. 26.

Prepare 1:2 dilutions by transferring 5 ml. of the 1:10 dilution to the Petri dish, the 1:10 and 1:100 dilutions being prepared as for milk. These dilutions will suffice for normal butter samples. A 1:1 dilution is not recommended because it is difficult to obtain a satisfactory dispersion of fat in the plate and because the yeast and mold clumps are not completely disintegrated.

3. MEDIA

Use a potato dextrose agar or a medium giving comparable results.

COMPOSITION OF POTATO DEXTROSE AGAR

Agar (best quality, not oven-dried).....	1.5 per cent
Glucose	2.0 per cent
Infusion from 200 gm. of potato.....	1,000 ml.

Reaction adjusted with tartaric acid at the time of pouring
to pH 3.5 ± 0.1

PREPARATION OF POTATO INFUSION

Place 200 grams of sliced potato, previously peeled or scraped, in 1,000 ml. of distilled water; boil for one hour and strain through a double thickness of clean toweling; restore to original volume. Add glucose and agar and dissolve in the autoclave at 115° C. Filter, distribute in flasks in definite quantities (usually 100 ml.) and sterilize as in Section B, III, 3, p. 24.

4. ADJUSTMENT OF REACTION

Prior to pouring plates, adjust the reaction to $\text{pH } 3.5 \pm 0.1$ with 10 per cent tartaric acid. Adjust only the amount of medium needed for samples which are to be plated the same day, since remelting the acidified medium may destroy its solidifying properties.

Adjustment of reaction may be made colorimetrically as follows: Remove 1 ml. of the sterile tartaric acid stock solution (10 gm. of tartaric acid crystals U.S.P. dissolved in 100 ml. of distilled water). Adjust the reaction of 5 ml. of medium, using the same procedure as given in Section B, III, 2, p. 23, except that the indicator used is brom phenol blue. Each ml. of dilute tartaric acid used will represent the number of ml. of stock solution required to be added to 100 ml. of medium. The amount of 10 per cent tartaric acid to be used will vary according to the buffering qualities of the medium. Usually from 1.0 to 2.6 ml. per 100 ml. of media are required.

Electrometric procedures for adjusting reactions are preferable.

The reaction of each batch of medium must be adjusted, but it is unnecessary to check the pH of each bottle of the batch before pouring.

5. INCUBATION

Incubate the plates in an inverted position for 5 days at 21° or 25° C.

It is advisable to examine the plates for mold and mycoderma growth at the expiration of 3 days. If molds or mycoderma are developing in large numbers count the plates at 3 days and make a recount on the 5th day. The count obtained on the 5th day is the desired count.

6. COUNTING

Count the number of mold and yeast colonies without the aid of a counting lens.

The count ratio between dilutions should not exceed 2.0.

When in doubt, decide the nature of a colony by a microscopic examination of the colony.

7. INTERPRETATION OF COUNTS

The yeast and mold count is an index of (1) plant sanitation, (2) inefficient pasteurization, and (3) carelessness of employees. It is not an index of the quality of raw materials used, nor is it an accurate index of the keeping quality of the butter.

IV. MOLD MYCELIA IN BUTTER

*Official Method*⁸ (First Action), Association of Official Agricultural Chemists

1. REAGENT

Gum solution—Make up 1 liter of a 0.75 per cent solution of carob bean gum with 2 per cent of added formaldehyde as a preservative. (The dry gum may be conveniently added by first mixing it in 10 to 15 ml. of alcohol and stirring the mixture rapidly into the water. Gently heat the solution to boiling to drive off the alcohol and air and continue heating 25 to 30 min. Add formaldehyde on cooling.) Use the clear supernatant solution free from cells, left when the cellular elements in gum gradually settle out. (A similar solution made with gum tragacanth may also be used for this purpose.)

2. APPARATUS

(a) *Compound microscope*—Equipped with good objectives and oculars, giving a magnification of about 90, 180, and 500 diameters. For convenience of use, the lenses should be adjusted so as to be parfocal. A mechanical stage is highly desirable. It is essential that the combination giving the low magnification be capable of adjustment to give an area of the field of view of 1.5 sq. mm: (a circle whose diameter is 1.382 mm.). With the higher powers the working distance must be ample to allow free use of the blood counting cell.

(b) *Drop-in-cross-ruled disc*⁹—For estimating lengths of mold filaments, an ocular drop-in-disc cross ruled in sixths of the ocular diaphragm opening is desirable.

(c) *Blood counting cell*—Ruled in the Thoma or the old Neubauer system of rulings. The so-called “improved” system of Neubauer is not suitable for this purpose.

(d) *Howard mold-counting cell*¹⁰—Constructed like a blood counting cell but with unruled central disc about 19 mm. in diameter.

3. PROCEDURE

Make a careful examination of the surface of the butter and note any visible mold. To remove possibility of contamination by any invisible surface mold, scrape off and discard $\frac{1}{8}$ in. of the surface, after which take the sample from the exposed surface.

Weigh out 1 gm. of butter by means of a $\frac{1}{4}$ teaspoon measure. Measure out 7 ml. of the hot gum solution and, with spoon held

bottom-side-up over a 50 ml. beaker, pour 2 or 3 ml. of the hot solution over the spoon to loosen the butter. Use the remainder of the hot solution to complete the rinsing of the fat from the spoon. Stir until the mixture is uniform and fat globules are 0.1–0.2 mm. in diameter. (The amount of stirring necessary must be determined by experience.)

Mount a portion of the mixture on the mold-counting slide and estimate the amount of mold present as directed under 4. Report no field positive unless the combined length of the two longest filaments exceeds $\frac{1}{6}$ of the diameter of the field.

Alternate staining procedure—Add 1 or 2 drops of 5 per cent crystal violet solution to the gum-butter mixture after butter is melted. Mix preparation thoroughly and prepare slide as directed.

4. MOLDS

Clean the special Howard cell so that Newton's rings are produced between slide and cover glass. Remove cover and place a small drop of the well mixed sample upon the central disc; using a knife blade or scalpel, spread the drop evenly over the disc, and cover with the glass so as to give an even spread.

It is of the utmost importance that the drop be taken from a thoroughly mixed sample and spread evenly over the slide disc. Otherwise, when the cover slip is put in place, the insoluble material, and consequently the molds, may be more abundant at the center of the mount. Avoid using a drop that is much greater than is sufficient to fill the space between the center disc and the cover slip. Discard any mount showing uneven distribution, absence of Newton's rings, or liquid that has been drawn across the moat and under the cover-glass.

Place this slide under the microscope and examine with such adjustment that each field of view covers 1.5 sq. mm. This area, which is of vital importance, may frequently be obtained by so adjusting the draw-tube that the diameter of the field becomes 1.382 mm. When such adjustment is not possible, it is sometimes necessary to have a mechanic make an accessory drop-in ocular diaphragm with the aperture accurately cut to necessary size. The diameter of the area of the field of view may be determined by the use of a stage micrometer, or by employing the rulings on the blood counting cell. In order to use the latter method it is necessary to bear in mind that a square whose diagonal is 1.382 mm. has sides of about 0.977 mm.

Hence the millimeter scale on the blood counting cell can be used by making such an adjustment that the circle of the field of view cuts off the necessary amount from each corner of the millimeter ruled square. When the instrument is properly adjusted, the quantity of liquid examined per field is 0.15 cu. mm. (0.00015 ml.).

From each of two or more mounts examine at least 25 fields taken in such a manner as to be representative of all sections of the mount. Observe each field, noting the presence or absence of mold filaments and recording the result as positive or as negative, as the case may be. Calculate the proportion of positive fields from the results of the examination of all of the observed fields and report as percentage of fields containing mold filaments.

V. BURRI SLANT METHOD ¹¹ (TENTATIVE)

The method consists of picking small portions of butter with a platinum needle and spreading each portion on the surface of a dry agar slope (see Section G, I, 3, p. 89).

The butter to be examined is placed in a sterile Petri dish and brought to approximately 21° C. This is temperature that yields a reasonably firm body with most types of butter so that small amounts can be easily picked.

The butter may come from a surface that has been exposed for some time, a freshly cut surface, or a freshly broken surface. Under a microscope giving approximately a 6X magnification very small amounts are picked with a flamed platinum needle. The use of a binocular microscope is advisable since it aids in keeping the portions of butter uniform in size and also in actually picking each portion rather than scraping it from a relatively large area; the latter point is important in obtaining information on the distribution of organisms. A needle of platinum, rather than some other material, is used because of the greater resistance to changes during heating. Each portion of butter is spread on the surface of a dry agar slope, care being taken to distribute it evenly. Ordinarily 25 portions of butter are picked from a sample. Larger or smaller numbers may be used if either more or less detail in the results is desired.

The slopes are incubated at 21° C. for 4 to 5 days and then the colonies are counted. Other incubation conditions can be used for special purposes but temperatures appreciably higher than 21° C. prevent the growth of certain organisms important in butter, while temperatures appreciably lower often result in slow growth. The

maximum number of colonies that can be counted satisfactorily on a slope is approximately 100, depending somewhat on the size of the colonies and the tendency for them to grow together. In some instances it is an advantage to examine the tubes showing extensive growth before the usual incubation period has expired. Numbers exceeding 100 per tube often can be estimated with considerable accuracy, but under these conditions it is probable that many colonies fail to develop.

With practice reasonably constant amounts of butter can be picked from the surface of the butter if the consistency of the butter is kept constant by bringing it to a temperature of 21° C. Where temperatures are higher than this, there is a tendency to pick larger amounts of butter. Determine the collective weight of 15 to 25 portions and by this means find the average weight of each portion. In the original work the authors found that each portion weighed approximately 0.05 mg. or 1/20,000 gram. By multiplying the number of colonies found on each slope by 20,000 they found the number of colonies per gm. of butter. The counts found are somewhat lower than the counts found by plating melted butter directly, but this technic permits the examination of specific portions of any given surface of the butter.

Simple modifications of standard agar may be made for the detection of lipolytic¹² or proteolytic^{13, 14} organisms.

VI. THE MICROSCOPIC EXAMINATION OF BUTTER¹⁵ (TENTATIVE)

1. GENERAL OUTLINE OF PROCEDURE

(a) Melt carefully a representative sample of butter by heating to 45° C.

(b) Centrifuge 10 ml. of the melted butter in a separatory funnel until the serum is separated from the fat.

(c) Draw off the serum.

(d) Spread 0.01 ml. of the thoroughly mixed serum measured with a 0.01 ml. pipette (Section C, Fig. IX, p. 44) over a definite area (from 1 to 9 sq. cm.) on a microscope slide and allow to dry.

(e) Stain as in the microscopic count for milk. (See Section C, IV, 4, p. 53).

(f) Examine under the microscope for the general character of the flora.

(g) If an estimate of the number of organisms per ml. of butter is desired, determine the number per microscopic field of the serum

and then calculate the number per ml. of serum and finally divide by 9 to determine the number per ml. of butter.

Following the suggestion in Section C, IV, 1, p. 51, that in the microscopic count of the bacteria in milk, cleanliness of glassware is more important than sterilization, the regular procedure has been to clean thoroughly, between samples, the equipment with which the butter or serum comes in contact and then, between periods of use, to treat such equipment with a cleaning solution (Section B, IV, 6, p. 28).

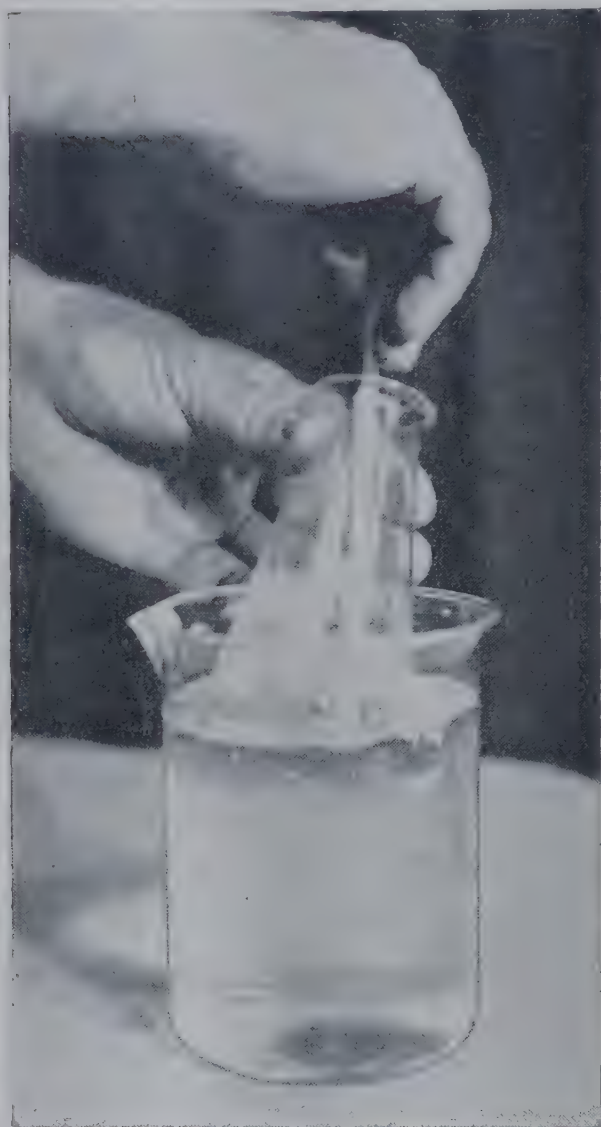


FIGURE XX Melting the Sample of Butter

2. DETAILED OUTLINE OF PROCEDURE

(a) The methods of sampling butter given above (Section J, I, p. 110) are satisfactory for the procedure under consideration and accordingly are followed. The entire sample of butter, or enough of it to yield 10 ml. of the melted product for centrifuging, is put into a beaker so that it can be readily melted and mixed. The melting and mixing are carried out by placing the beaker in a water bath at a temperature of from 50 to 55° C., and slowly agitating the butter, using a thermometer as a stirring rod, until the temperature reaches 45° C. (Figure XX).

(b) Ten ml. of the melted butter at a temperature of 45° C. are transferred to a dry 30 ml. separatory funnel (Figure XXI)* using a pipette that has

* The type of separatory funnel originally used was the one listed in apparatus catalogs that seemed best suited to the requirements. If a special separatory funnel is secured there is no need of including a constriction at the top, and the shoulder above the stop-cock should have a gradual slope so that there will be less tendency for the sedimented curd, etc., to stick to the glass when the stop-cock is opened; a large bore in the stop-cock is an additional advantage. Figure XXII shows a special separatory funnel of 30 ml. capacity that has been found satisfactory.

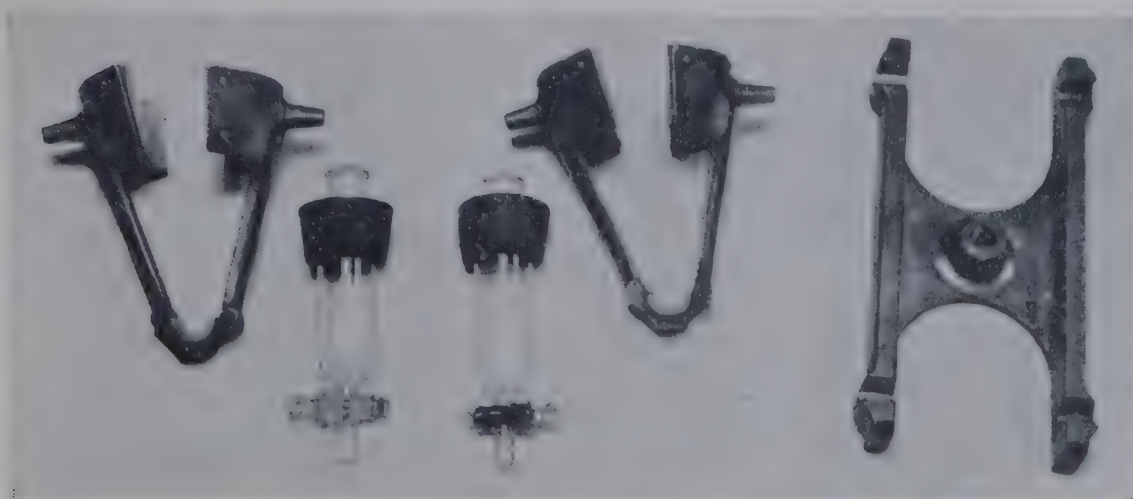


FIGURE XXI—Separatory Funnels, Funnel Supports and Centrifuge Head

been warmed by passing it through a flame or by sucking up and discharging sterile water heated to 55° C. The size of the portion centrifuged is of no great importance but a standard volume seems advisable and one of 10 ml. has some advantages over smaller amounts. These advantages are: (1) Enough serum collects to permit the removal of the serum without including any considerable percentage of fat, and (2) the volume of serum obtained provides ample material for plating in case the microscopic picture makes this advisable.

Centrifuging is carried out using a funnel support (Figure XXI). A rubber stopper around the upper part of the funnel rests against the wall of the support and the funnel is carried in this way. The assembled funnels, funnel supports, and centrifuge head clamped to the top of a ring stand are shown in Figure XXIII. Very little funnel breakage has been encountered and most of this has involved the tip of the funnel which projects into the lower portion of the support.

The procedure may be simplified when the usual equipment is not available by centrifuging the melted butter in an inverted, stoppered test tube. Place the inverted test tube in ice water to solidify the fat. On removal of the stopper, the serum is collected in a small test tube for examination.

The centrifuge should throw out the serum completely without causing a separation in the serum itself. When centrifuging has been done properly the fat layer will be clear and the serum layer uniform

throughout (Figure XXIV). With a centrifuge in which the stop-cocks of the funnels were $14\frac{1}{2}$ inches apart with the machine in operation, centrifuging 1 minute at a speed of 1,000 r.p.m. was found to be entirely satisfactory for the types of butter used. Samples of butter of different characters behave differently, and some types are easily over-centrifuged so that there is a separation in the serum. When this occurs a solid pack of material at the bottom of the funnel may make it very difficult to start a flow through the stop-cock and it becomes imperative to remove the serum very completely,

and then thoroughly mix it, if a representative portion is to be obtained for spreading on the slides. In general, the centrifuging of butter made from a poor quality of cream must be carefully done or there will be a separation in the serum; presumably, this is due to the content of particles of casein or some casein derivative that sediments readily. Where butter is made from good quality sweet cream, the centrifuging may exceed that suggested either in time or speed without any undesirable effect.

(c) Immediately after centrifuging, the funnel is removed from the centrifuge and the serum drawn off (Figure XXV). The serum should be removed slowly so that the plane between the fat and serum is kept as flat as possible. If the serum is drawn off rapidly the portion along the wall of the funnel tends to lag and the fat is pulled down to the stop-cock when there is still a considerable percentage of the serum in the funnel. The serum is removed as completely as possible without including objectionable amounts of fat.

A short test tube (Figure XXV) makes a very satisfactory container in



FIGURE XXII—Special Separatory Funnel for Centrifuging Melted Butter

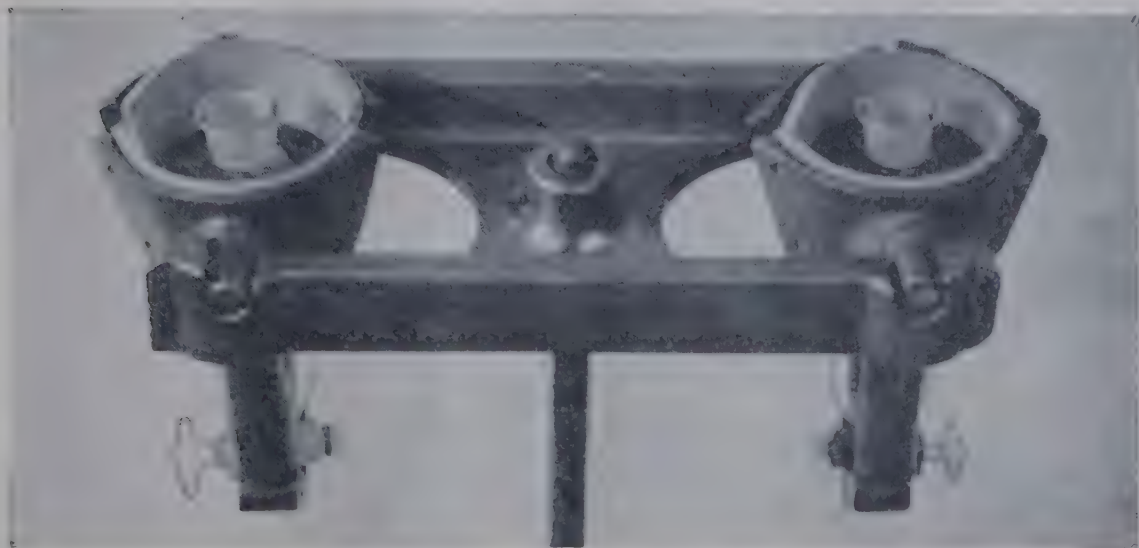


FIGURE XXIII—Assembled Funnels, Funnel Supports and Centrifuge Head Clamped to the Top of a Ring Stand

which to collect the serum since portions of the serum can be easily removed from it with a 0.01 ml. pipette (Section C, Fig. IX, p. 44).

(d) The collected serum is thoroughly agitated, and portions are removed to microscope slides with a 0.01 ml. pipette. Ordinarily 0.01 ml. of the serum is



FIGURE XXIV—Centrifuged Butter Showing Clear Fat Layer and Uniform Serum Layer



FIGURE XXV—Removing the Serum from the Separatory Funnel

spread over 1, 4, 5, or 9 sq. cm. If the area is larger than 1 sq. cm. sterile skim milk, free from stainable bacterial cells, or water is added with a loop to facilitate even distribution. The spreading is conveniently done with a transfer needle bent at an angle of 90 degrees. When skim milk or water is to be used it should be put near the 0.01 ml. of serum and thoroughly mixed with the serum before an attempt is made to cover the desired area.

The area over which the 0.01 ml. of serum should be spread depends on the bacterial content of the butter and the information desired. If a study of the morphologic types of organisms present is the main consideration it is an advantage to have the organisms rather numerous in each field while if an estimate of the number present is desired the organisms should not be so numerous that counting is difficult. With butter having a very high bacterial content, counting may be facilitated and the calculation simplified by spreading the 0.01 ml. of serum over an area of 9 sq. cm.

A card with the desired area laid out in black and surrounded by a broad red border is a great aid to making spreads that are reasonably exact in area and reasonably uniform in thickness. If a portion of the background is white the desired results are not so easily secured.

The slides are dried rapidly on a level surface, protected from insects and dust.

(e) The staining procedures described in Section C, IV, 4, p. 53, are satisfactory for staining preparations of the serum secured from butter.

(f) The examination of the stained smears under the oil immersion lens shows the general morphologic types and the general number of organisms present.

(g) The number of organisms per ml. of butter may be estimated by (1) determining the average number per field, counting as many fields as seems desirable for the purpose in mind; (2) calculating the number per ml. of serum from the area over which 0.01 ml. of serum was spread and the diameter of the microscopic field; and (3) dividing by 9 (see next page) to change from a basis of 1 ml. of serum to a basis of 1 ml. of butter. Steps 2 and 3 may be combined for routine work.

3. TRANSPOSING COUNT FROM SERUM TO BUTTER BASIS

The results obtained in the examination of butter serum are more readily usable and more easily compared with the results obtained from other dairy products if they are changed to a basis of 1 ml. of butter. Accordingly, a factor for making such changes has been developed. The organisms present in the butter fat may be ignored as investigation¹⁵ has shown that practically all of the bacteria are present in the serum. Determinations of the amount of moisture present in lots of acceptable butter showed that the volume of serum obtainable from 10 ml. of butter quite regularly equalled 1.1 ml. or approximately 1/9 of the volume of the butter. Accordingly, the bacterial count per ml. of serum should be divided by 9 to change the serum counts to a basis of 1 ml. of butter. Where the serum is spread over 9 sq. cm. the bacterial count is determined directly as the count per ml. of butter; in that 1 ml. of serum has been extracted from 9 ml. of butter fat.

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K. TESTS FOR STERILITY OF DAIRY CONTAINERS, UTENSILS, AND EQUIPMENT

Periodical examination for sterility of containers, utensils, and equipment in which dairy products are to be handled or stored is an important duty of the control official.

It is desirable that tests be made at intervals of not more than a month, concentrating the work, however, where previous tests indicate the worst conditions are to be found.

The term "sterility" is used in this section in its true sense as applied to laboratory equipment and procedures. However, as applied to containers and equipment the term "sterility," for want of a better word, is used to mean freedom from bacteria within practical commercial limits in accordance with standards herein suggested in connection with the interpretations of results.

I. RINSE METHODS

The rinse method is particularly applicable to containers such as bottles and cans, but may be applied to equipment.^{1, 2}

1. SAMPLING

Care should be exercised in handling containers to avoid possible contamination by the fingers or through permitting milk or water to drip into the empty containers as by passing them under dripping bottle filler valves prior to closing or capping. Paper containers collected as they pass through the bottler should be collected before any water or milk is introduced into the bottle fillers. Glass bottles to be capped for testing should be capped mechanically in accordance with plant routine, following the procedure given in Section K, I, 3A, p. 127. At least 3 bottles should be selected for examination. A visual examination of glass bottles may reveal some with a greasy appearance. Include one or more such bottles among the samples to be tested. Also examine at least two milk or milk products cans. An inspection of cans having seams is quite likely to show some with seams broken. Include one or more of these cans among those rinsed.

2. APPARATUS AND MATERIALS

Apparatus—Sterile 10 ml. (or 11 ml.), 1 ml. (or 1.1 ml.), and 0.1 ml. pipettes, sterile Petri dishes, and standard agar are used in this work (Section B, II and III, pp. 16–25). Sterile non-toxic tap water or sterile buffered distilled water in marked test tubes containing 10 ml. and in marked dilution bottles containing 100 ml. or in other suitable containers, is also required. If sterile tap water is used, it should be determined by test that the sterilized tap water will not destroy bacteria within a period of 2 hours, or longer if samples are to be shipped. Fill the marked test tubes and marked dilution bottles with non-toxic tap water or buffered distilled water to such level as determined by trial that after sterilization in an autoclave at 121° C. (15 lbs. steam pressure) for not less than 20 minutes and cooling to room temperature, they will be filled to the mark. An alternate tentative procedure requires the use of standard nutrient broth (see below) instead of tap water or sterile buffered distilled water, and the use of a sterile syringe^{2a} of at least 20 ml. capacity, or a gravity flow or siphoning device.³

Buffered distilled water—The purpose is to secure a solution that is non-toxic to bacteria. Prepare a stock solution by dissolving 34 gm. of potassium di-hydrogen phosphate (KH_2PO_4) in 500 ml. of distilled water, adding about 175 ml. of a normal sodium hydroxide solution, and dilute to 1 liter with distilled water. Adjust this solution to pH 7.2 (See Section B, III, 2, p. 23). Dilute 1 ml. of this stock solution to 800 ml. with boiled and cooled distilled water. If the solution is to be used in rinsing containers which may contain residual chlorine, add 4 ml. of a 0.1 N sodium thiosulfate solution to the 1 ml. of stock solution of KH_2PO_4 before diluting it to 800 ml. with boiled and cooled distilled water in preparing the buffered distilled water solution.

Sodium thiosulfate solution approximately 0.1 N—Prepare by dissolving 25 gm. of crystallized sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3 \cdot 5 \text{H}_2\text{O}$) in recently boiled and cooled distilled water, filter and dilute to 1 liter with such water. Keep in a cool dark place such as a refrigerator.

Standard nutrient broth—Prepare in accordance with the instructions given for preparing standard nutrient broth in Section B, II, 13, p. 22.

Water—See Section B, II, 8, p. 21. As stated in this section, use distilled water in making up all reagents; use non-toxic distilled water or tap water for rinsing and dilution where plating follows within 30 minutes; use buffered distilled water when rinse waters are to be iced and held for periods longer than 30 minutes before plating.

3. PROCEDURE FOR EXAMINING CAPPED MILK BOTTLES AND PAPER CONTAINERS

A. METHOD OF RINSING

In testing milk bottles 20 ml. of sterile tap or buffered distilled water are introduced into each bottle to be tested, and each bottle is capped mechanically by plant equipment following the routine plant method, using stock caps. Do not allow the bottle to travel under the filler valves. First wipe the skirt of the capper with a piece of sterile gauze, waste one cap, place the bottle directly under the capper and apply a cap. Grasp the bottle by the neck while upright and swing it 25 times in a small circle to rinse the bottle thoroughly. Then, holding the bottle in a horizontal position, shake vigorously back and forth 25 times in the direction of the long axis of the bottle for a distance of about 8 in. and at the same time rotate the bottle slightly at the end of each stroke in order to make 8 complete rotations in the course of the shaking. The intention is to rinse every portion of the interior of the bottle and cap thoroughly.

Where required, transfer the solution by sterile pipette to a sterile sample vial for transfer to the laboratory. If it is necessary to pour the solution into a sterile vial, the pouring lip of the bottle should be first carefully flamed. The samples should be kept at 50° F. or lower during transit to the laboratory. In testing bottles that may contain residual chlorine, use sterile tap or buffered distilled water with 0.1 N sodium thiosulfate solution added as previously directed, or use the tentative nutrient broth method which follows.

B. METHOD OF PLATING

When low counts are expected and experience shows that it is desirable, distribute 10 ml. of the rinse water approximately equally among 3 agar plates. When the expectation is that over-

crowded plates will be produced by this procedure, transfer 1 ml. of the rinse solution by means of a sterile pipette to each of two Petri dishes and 0.1 ml. portions to each of two other Petri dishes. Pour 10 ml. portions of standard agar directly upon the rinse solution in the Petri dishes. Rotate each Petri dish in such a manner as to mix thoroughly the rinse solution and agar. Not more than 15 minutes shall elapse between the time the bottles are rinsed and the time the last plate is poured. If plating cannot be finished as promptly as this, the sample of rinse solution shall be cooled rapidly to a temperature not exceeding 10° C. (50° F.), and maintained at such temperature until plated. Incubate plates at the temperature used for Standard Plate Counts for 48 hours. Count as in the case of plates prepared from milk. The sum of the number of colonies that develop on each of the 3 plates receiving 10 ml. of rinse solution multiplied by 2, 10 times the sum of the number of colonies on the two plates receiving 1 ml. each of rinse solution, or 100 times the sum of the number of colonies on the two plates to which 0.1 ml. each was added, equals the estimated number of colonies per bottle.

A tentative alternate method is first to cap the empty sterile bottle and then inject 20 ml. of standard nutrient broth by means of a sterile needle through the bottle cap.³ Afterward proceed exactly as when sterile buffered distilled water is used except that this method is for use only when the plating is done immediately. This method has the advantage of introducing a medium that is favorable to bacteria and that will neutralize any residual chlorine that may be present in bottles sterilized by chlorine solution.

When the containers are practically sterile, a still more sensitive test is to rinse 50, 100, or even more containers with standard nutrient broth placed in the container with a sterile pipette or preferably injected through some protected spot near the top of paper containers or through the paper caps of glass bottles. The injection may be accomplished with a sterile syringe^{2a} containing at least 20 ml., or a siphon or gravity flow device.³ When siphons or gravity flow devices are used the broth is introduced into the container from a sterile supply in a flask or similar container by means of sterile tubing attached to a sterile needle. The amount of broth injected with the syringe is usually 20 ml. When siphoning or gravity flow devices are used 30 to 40 ml. of broth may readily be used. Shake the containers vigorously and then incubate the containers at the

temperatures used for Standard Plate Counts for 48 hours. If incubator space is limited the containers may be held at room temperature. At the end of 48 hrs. note the number of containers in which the broth remains sterile. The examination of the broth for cloudiness or other evidence of bacterial growth is sufficient. Report the results in percentage of sterile containers.

C. INTERPRETATION OF RESULTS

Quart bottles developing not more than 1,000 colonies, pint bottles developing not more than 500 colonies, and half-pint bottles developing not more than 250 colonies as determined by the method described above are generally considered satisfactory. Counts from poorly sterilized containers usually greatly exceed the figures given. However, it has been found by experience that properly sterilized containers will meet an even more severe standard than this. A standard of not more than 100 bacteria per bottle regardless of size can be met readily when efficient methods are used for washing and sterilizing bottles, and low count caps and machines with clean capping heads are used. Paper milk containers usually comply with the latter standard without difficulty.

Supplementary examination of caps by the following procedure will aid in determining the cause of high counts.

4. PROCEDURE FOR EXAMINING BOTTLE CAPS, HOODS, AND CLOSURES

A. METHOD OF EXAMINATION

It is advisable to test closure surfaces that come in contact with the milk along with the bottles whenever practicable. When and if it becomes necessary to test these products for sterility independently of the bottles, use the following procedure: Sterilize milk bottle blanks as shown in Figure XXVI* in an autoclave with kraft paper, metal foil, or other suitable covering sealed over the mouth. Pour about 10 ml. of melted standard agar into one of these blanks held with the bottle cap seat up, then, using sterile forceps, apply the cap to be tested printed side out, pressing it firmly into place without contaminating the inside surface. A convenient method of doing this is to use a sterile wooden plug.† As

* Obtainable from manufacturers of milk bottles.

† Obtainable from manufacturers of milk bottle caps.

soon as the cap is in place, quickly invert the blank, shake gently, and let it stand on a level table until the agar hardens. Then incubate the blank in an upright position at the temperature used for Standard Plate Counts for 48 hours. After incubation warm the blank gently to soften the edge of the agar in contact with the glass. Carefully remove the cap and the adhering layer of agar, and count the colonies on the agar.

Hoods and closures may be examined by placing one or more hoods or closures inside up in a sterile Petri dish. It may be necessary to use 150 ml. dishes for large skirted closures. Then pour 3 to 5 ml. of melted standard agar into each closure. Shake

Disc cap _____
Agar _____

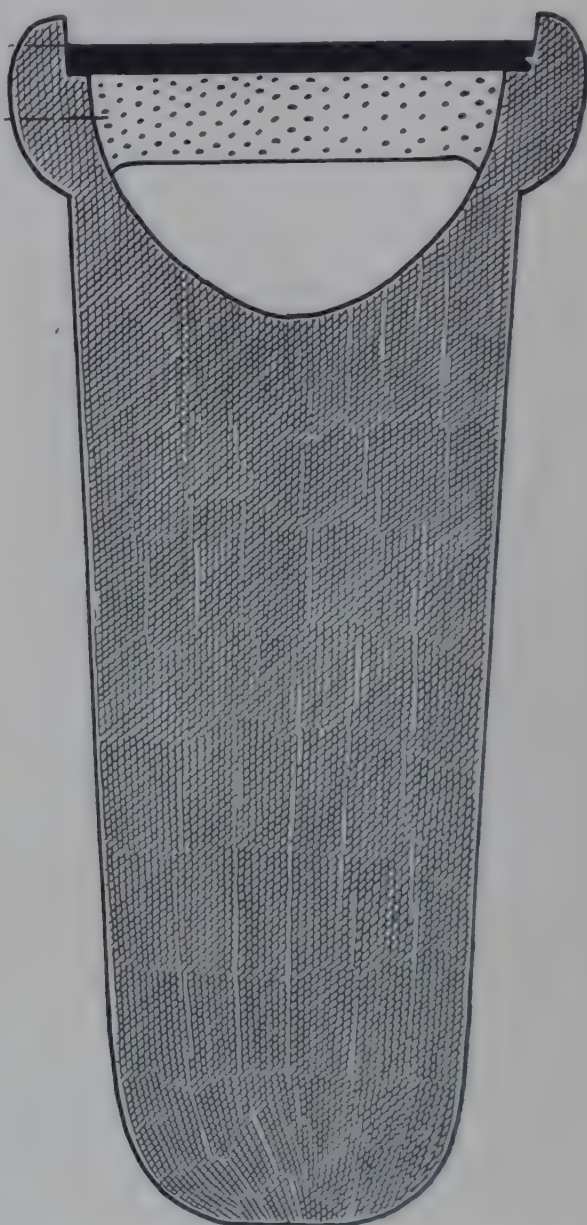


FIGURE XXVI—Milk Bottle Blank, Cap and Agar Disc

gently using sterile forceps. Cover the dish and incubate at the temperature used for Standard Plate Counts for 48 hours. Count the number of colonies per hood or closure.

B. INTERPRETATION OF RESULTS

Counts of not more than 10 per bottle cap, hood, or closure are considered satisfactory.

5. PROCEDURE FOR EXAMINING MILK, CREAM, AND ICE CREAM CANS ^{4, 5}

A. METHOD OF RINSING

Introduce 100 ml. of sterile tap water, sterile buffered distilled water, or sterile standard nutrient broth into the can to be tested. Tightly replace the can cover. If parchment papers are regularly used between can and cover, insert such paper in the usual manner. Some strength is required to rinse cans. A shaking machine has been devised, but is too large to be transferred rapidly from plant to plant ⁶ (Figure XXVII).

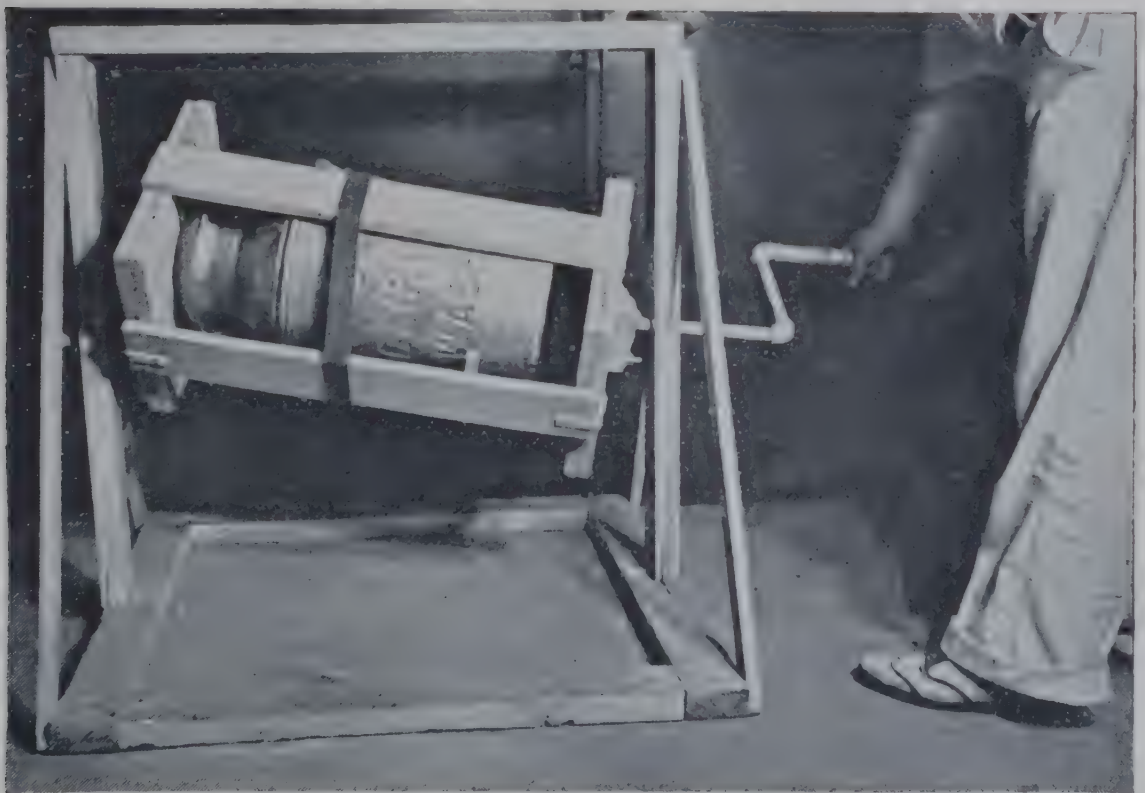


FIGURE XXVII—Home-made Machine Used for Rinsing Cans Mechanically

If hand shaking is used, lay the can on its side on the floor. Grasp the can under the cover with one hand and under the uppermost side of the bottom rim with the other and shake rapidly back and forth 10 times through a distance of about 18 in. Replace the can on the floor, giving it a quarter turn, and repeat the shaking process. Repeat the shaking twice more after giving the can quarter turns in the same direction. The can has then been shaken 10 times in each of four positions. Pour the rinse solution back into the container from which it was taken or collect it in some other sterile container for transfer to the laboratory.

B. METHOD OF PLATING

By means of a sterile pipette transfer 1 ml. of the rinse solution to each of two Petri dishes and 0.1 ml. portions to each of two other Petri dishes. Pour, incubate, and count the plates as outlined above, observing the usual precautions. The number of colonies on each plate, multiplied by 100 for the plates to which 1 ml. was added, and by 1,000 for the plates to which 0.1 ml. was added equals the estimated number of colonies per can and cover.

C. INTERPRETATION

Cans and covers developing not more than 40,000 colonies per 40 quart can, 20,000 per 20 quart can, 10,000 per 10 quart can, or 5,000 per 5 quart can as determined by the method described above are generally considered to be satisfactory. More stringent standards can readily be met where seamless cans and efficient can washers and sterilizers are used.

Those interested in more detailed information about the cleanliness of cans may adjust the pH of the media to correspond with that of the material in can, and use differential counting to determine the presence of proteolytic types of bacteria.¹²

6. RINSE METHOD FOR LARGER EQUIPMENT

A. METHOD OF RINSING

The sterility of larger dairy equipment may be determined by procedures adapted from the procedure used for metal cans (see Section K, I, 5, p. 131). Use appropriate amounts of sterile water, or where large quantities of water are needed, tap water may be used if this is known to contain not more than small numbers of bacteria capable of growth at the incubation temperature used for

the agar plates. In case unsterilized tap water is used, make control plates and subtract the number of colonies found in the tap water from the number found in the rinse water.

B. INTERPRETATION

Large, well cleaned dairy equipment that yields counts from rinse waters indicating less than one organism per ml. of capacity may be accepted as satisfactory, but well cleaned equipment will normally yield much lower counts than this.

C. USE OF MILK AS AN INDICATOR OF CONTAMINATION

A tentative method for determining the sterility of pipe lines, coolers, and bottle fillers in milk plants is to sample the milk with a sterile thief or pipette directly from the pasteurizing vat just before the milk pump is started, and then to collect a sample of milk in a sterile container from one of the bottle filler valves before bottling begins. The first bottle may be taken as a sample, but in case a positive result is obtained, this raises the question as to which was not sterile, the equipment or the bottle. Make Standard Plate Counts and coliform tests on these samples in accordance with methods previously described.

D. INTERPRETATION

When the Standard Plate Count of the second sample exceeds the first by 100 per cent plus 2,000, the result may be considered as indicating improper sterilization of the equipment. The sample from the pasteurizer should show no coliform organisms in 1 ml. portions. After determining this to be true, the presence of coliform organisms in 1 ml. portions of the sample of milk from the filler valve is an indication either of unsatisfactory sterilization or of recontamination of some portion of the equipment traversed by the milk between sampling points. This is a more sensitive test than the test for increase in count.

II. CONTACT PLATE METHOD ^{7, 8} FOR DETERMINING THE NUMBER OF BACTERIA PRESENT ON FLAT SURFACES

The contact plate method is particularly applicable to testing the sterility of equipment such as the inner surfaces of pasteurizers or large containers having relatively flat surfaces such as weigh vats, some types of pasteurizers, side walls and covers of pasteurizers, coolers, paperboard blanks, rectangular paper ice cream containers, and the like.



FIGURE XXVIII—Method of Applying Contact Plate to a Flat Surface Such as an Ordinary Plate



FIGURE XXIX—Contact Plate Showing Colonies Developed After Application to a Reasonably Clean Surface. The Dark Area Indicates a Pinpoint Defect in the Tin Plating of the Can Cover.

1. APPARATUS AND MATERIALS

Rubber suction cup, sterile Petri dishes, sterile No. 2 ($3\frac{7}{16}$ in. in diameter) can covers whose concentric ridges have been flattened,* and standard agar are used in this technic.

A tin cover is placed in a Petri dish so that the top of the cover faces the bottom of the Petri dish. After sterilization, the Petri dish is inverted and the bottom of the dish raised by the suction cup; 16 to 17 ml. of agar are pipetted into the tin cover; the bottom of the dish is replaced; and the agar allowed to harden. The Petri dish is stored in an upright position at a low temperature (about 15° C.) until used.

2. PROCEDURE FOR EXAMINING FLAT SURFACES

A. METHOD OF APPLYING CONTACT PLATE

Raise the top of the Petri dish and remove the tin cover using the suction cup (Figure XXVIII). Press the contact plate lightly for approximately 4 seconds, against any selected spot on the surface to be examined. Return the contact plate to the Petri dish and incubate at the temperature used for Standard Plate Counts for 48 hours. Then remove the plate and count the colonies in the usual manner (Figure XXIX). In large equipment at least two spots should be examined. Do not hesitate to examine spots that may be most difficult to reach in cleaning or that look slightly greasy.

B. INTERPRETATION

It is only reasonable to expect that every portion of the surface show not more than 10 colonies per plate.

III. SWAB TEST FOR EQUIPMENT^{9, 10}

This is a tentative method to be used on equipment where, due to the irregularity of the surface or size of the equipment, neither the contact plate nor rinse method is applicable.

1. APPARATUS AND MATERIALS

Sterile Petri dishes, sterile 1 ml. pipettes, standard agar, sterile cotton swabs on stiff non-corrodible wire holders, sterile 15 mm. by

* Obtainable from Will Corporation, Rochester, N. Y.

100 mm. test tubes with cork or rubber stoppers, thin flexible sheet metal frames with openings 4 sq. in. in area, alcohol burner, and automatic shaking machine.

2. REAGENTS

Buffered distilled water—Prepare the phosphate buffer solutions as directed in Section K, I, 2, p. 126. Distribute the diluted solution in 15 by 100 mm. test tubes in amounts that will provide exactly 4 ml. after autoclaving for 30 minutes. Use cork or rubber stoppers in the tubes; cotton plugs are not satisfactory.

3. COLLECTING SAMPLES

The swab should be moistened in a freshly-opened tube of buffered dilution water and rubbed slowly and thoroughly over 4 sq. in. of the sterilized surface which may be delineated by holding against it a flamed sheet metal frame with 4 sq. in. opening. Rub the swab three times over every part of the surface. The swab should be replaced in the tube of buffered dilution water, and the tubes should be sent to the laboratory promptly, keeping them refrigerated while in transit.

4. LABORATORY PROCEDURE

A. METHOD OF PLATING

Shake the tubes for 10 minutes in an automatic shaking machine, using a lateral motion in order to wash adherent material from the swab into the buffered dilution water. Remove the swab, pressing it against the wall to expel as much moisture as possible.

Transfer 2 ml. of the dilution water to a sterile Petri dish. Add approximately 10 ml. of melted standard agar, mix, incubate at the temperature used for Standard Plate Counts for 48 hours, and count as in making a Standard Plate Count. Multiply the count by two and report as the plate count of organisms removed from an area of 4 sq. in.

B. INTERPRETATION

Counts in excess of 100 per 4 sq. in. of surface are considered unsatisfactory.

IV. RINSE TEST AS APPLIED TO ICE CREAM FREEZERS ¹¹**1. SAMPLING APPARATUS AND MATERIALS**

Same as for the rinse test (see Section K, I, 1-2, pp. 125-127) except that the sterile rinse solution should be put up in 100 ml., 200 ml., and 400 ml. portions.

2. COLLECTING SAMPLES

Use 100 ml. of sterile rinse solution for a 10 quart freezer, 200 ml. for a 20 quart freezer, and 400 ml. for a 40 quart freezer. If chlorine has been used for sterilizing the freezer, use a sterile rinse solution containing sodium thiosulfate.

Pour the sterile solution into the inlet of the assembled freezer, first making sure that the outlet gate is closed. Put the machine in operation for 2 minutes and then shut it off. Open outlet gate slightly, first allowing a small portion of the rinse solution to run to waste, and then catch a portion of the solution in a sterile container for transfer to the laboratory.

For continuous flow freezers, close the necessary valves to retain a portion of rinse water in the freezing chamber and pour 4 liters of tap water into the mix level inspection port or into disconnected sanitary pipe inlet of the assembled freezer. Operate the freezer for 30 seconds, stop and after wasting all water that stood in the discharge line collect a portion of rinse water from the discharge line in a sterile container for transfer to the laboratory. Unsterilized tap water may be used for the 1 liter rinse for 100 quart freezers, and the 4 liter rinse for continuous freezers, because it is impractical to sterilize these large quantities of rinses. However, a Standard Plate Count should be run on a sample of the water used, and this count, multiplied by the number of milliliters of rinse water should be subtracted from the total count of organisms rinsed from the freezer. It is necessary to use 4 liters of rinse water for continuous freezers, notwithstanding their small capacity, in order to protect moving parts against damage during rinsing.

3. LABORATORY PROCEDURE**A. METHOD OF PLATING**

Follow the method of plating and estimating counts described for the agar plate count under Section B, V-VIII, pp. 28-37. Prepare

dilutions that are likely to give plates with between 30 and 300 colonies. Dilutions as high as 1 to 10,000 may be necessary when this work is first undertaken.

B. INTERPRETATION

Freezers showing a total rinse count of not more than 5,000 for continuous flow freezers, 10,000 for the 10 quart capacity batch freezer, 20,000 for the 20 quart capacity, 40,000 for the 40 quart capacity, and 100,000 for the 100 quart capacity, are generally considered to be satisfactory.

V. DISINTEGRATION METHOD FOR MAKING BACTERIAL COUNTS FROM PAPER AND PAPER-BOARD USED FOR BOTTLE CAPS, HOODS, CLOSURES, CUPS, FIBER CANS, AND CONTAINERS^{13, 14} (TENTATIVE)

Health officials are interested in determining whether the paper and paper-board used for making milk bottle caps, hoods, and closures, single service milk containers, cups, fiber cans, wrappers, and containers for packaging or distribution of frozen desserts, cottage cheese, cream cheese, butter and other dairy products are manufactured under good sanitary conditions as reflected in the bacterial content of the paper or paper-board.

1. COLLECTION OF SAMPLES

Cut samples with sterile scalpels or other sterile cutting devices from rolls of paper or paper-board, sheeted stock, nested containers, blanks, or closure discs, taking at least 100 gm. of stock, and transfer with sterile forceps to sterile kraft wrappers or envelopes. Wrap sealed samples in heavy kraft paper to protect them from moisture and extraneous contamination during transit to the laboratory. Bottle caps, hoods, and closures may be transferred to sterile envelopes with sterile forceps or sampled directly from tubes or packages used in shipping these products.

2. APPARATUS AND MATERIALS

Sterile 10 ml. pipettes with ends cut to a bore of about 3.0 to 4.0 mm. (to permit passage of pulp), sterile Petri dishes equipped with porcelain tops glazed on outer surface, sterile water blanks, standard agar with no skim milk added, sterile scalpels, sterile scissors, sterile forceps, balances, disintegrator with covered sterile steel cup, and kraft wrappers or envelopes.

Two specially modified mixers of the type used at soda fountains have been found to be especially satisfactory for the disintegration of paper or paper-board. These are the Stevens mixers made by the Stevens Electric Company at Racine, Wis., and the cheaper and equally satisfactory Eskimo-Whiz-Mix made by the Bersted Manufacturing Company of Fostoria, Ohio. Stainless steel cups of 820 ml. capacity are furnished with the Stevens mixer, and similar cups of 1,100 ml. capacity with the Bersted Mixer.

3. PROCEDURE

A. METHOD OF MAKING ANALYSES

Use sterile scissors or other suitable cutting device, to cut the middle portion of the sample sheet into pieces of not more than $\frac{3}{8}$ in. in greatest dimension, collecting the pieces in a sterile Petri dish. Precautions should be taken while sampling against contaminating the paper through talking or sneezing. Place the Petri dish on the balance pan and, using sterile forceps to handle the stock and taking precautions to prevent chance contaminations, weigh out 5 gm. of the sample. Place 500 ml. of sterile water (see Section B, II, 8, p. 21) in the large sized sterilized mixer cup, add 5 gm. of paper or paper-board to this water and replace the cover. With the smaller sized cup use 300 ml. of sterile water and 3 gm. of paper or paper-board. Place the cup on the driving mechanism. Start in low speed, if available, for 15 seconds and then run on high speed for a total of 5 minutes. After the first $\frac{1}{2}$ minute of this 5 minute period turn off the motor, remove the cover and, if any pieces of paper or paper-board are found adhering to the wall of the cup above the water line, rinse them into the body of water by revolving the cup. Reëxamine at intervals until it is certain that no pieces remain on the sides.

Because only a few bacteria are found per gram of good quality paper-board such as is used in making bottle caps, closures, and milk containers, and because these are largely of spore-forming types that produce spreading colonies, it is difficult to make accurate counts and special precautions are needed. Protect plates against dust or other contaminations as even an occasional colony from such sources causes a large percentage error. Porcelain tops for Petri dishes are practically essential in restraining the growth of spreading colonies.

In analyzing high grade paper-board made from virgin stock, a

1:10 dilution is usually satisfactory. Using a 10 ml. sterile pipette with the large bore at tip, divide 10 ml. of the disintegrated paper or paper-board approximately equally among 3 sterile Petri dishes, equipped with porcelain tops. Pour 10 to 12 ml. of melted standard agar cooled to 41 to 42° C., directly upon the disintegrated stock in each of the Petri dishes. Rotate each Petri dish in such a manner as to mix thoroughly the disintegrated paper or paper-board and the agar. In analyzing board containing secondary stock, higher dilutions are usually necessary.

In the directions given, the pulp is concentrated as much as feasible in order that the number of colonies will be kept as large as 5 per plate if possible. This reduces the distribution and contamination errors.¹⁵ Some prefer ¹⁶ to distribute the pulp in 5 plates instead of 3, but this increases still more the need for taking precautions against dust contaminations.

Allow the agar to harden in the plates and then incubate at the temperature used for Standard Plate Counts for 48 hours. Count the plates as in making Standard Plate Counts of milk. The sum of the colonies on the 3 or 5 plates represents the number of colonies developing from 0.1 gm. of paper or paper-board stock.¹⁵ Multiply this figure by 10 and report the result as the number of colonies per gram of stock. Use a Quebec Colony Counter or other special lighting devices in counting plates in order to avoid overlooking colonies. Precautions should also be taken to avoid counting tiny air bubbles, grit or other particles as colonies.

B. INTERPRETATION

Paper or paper-board developing not more than 500 colonies per gram of disintegrated stock in three of the last four analyses of these products taken from different runs is generally considered satisfactory. This is the percentage compliance method preferred by some authorities ¹⁷ and does not involve averaging.

A standard of not more than 250 colonies per gm. calculated as the logarithmic average of the bacterial plate counts of the last four analyses of these products taken during a grading period is more severe than the above. This is the standard method of computation used in the 1939 *Milk Ordinance and Code* of the U. S. Public Health Service.

Where clean and sanitary, selected secondary stock filler is per-

mitted and specifically approved by a public health official for use in inside layers of heavy paper-board, e.g., in fiber cans for ice cream, these filled boards usually meet a standard of not more than 1,000 colonies per gm. of disintegrated stock.

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PART II. MICROBIOLOGICAL METHODS FOR THE EXAMINATION OF FROZEN DESSERTS AND INGREDIENTS OF FROZEN DESSERTS

LABORATORY, AND FOOD AND NUTRITION SECTIONS

American Public Health Association

INTRODUCTION

The directions for the microbiological examination of frozen desserts include methods for examining all types of frozen desserts and the ingredients used in making them. The term frozen desserts is used in this report to include plain ice cream; ice creams containing nuts, seeds, fruits, or miscellaneous confections with or without reinforcing flavoring materials; ices, ice sherbets, milk sherbets, frozen custards, and frozen milks; and all frozen desserts made in imitation of ice creams. Moreover, the directions are applicable to the examination of mousse, frappe, spumoni, coupe, sundae, bombe, bisque, parfait, frozen puddings, au fait and other miscellaneous frozen novelties, specialties, and confections of a similar character. The estimated bacterial content shall be reported preferably in terms of the "Standard Plate Count" hereinafter more fully explained.

These directions are designed primarily for the examination of frozen desserts as retailed to the consumer or as sold at wholesale, but they may be applied for control purposes to the unfrozen, partially frozen, or melted (defrosted) mix at any time after its preparation.

The methods given for the microbiological examination of the ingredients of frozen desserts include methods for examining evaporated, condensed and dry milks, flavoring extracts, fruits, nuts, sweetening agents, and eggs and egg products. Directions for making sediment tests of frozen desserts and of ingredients of frozen desserts are also given.

Methods for the examination of cream and butter are given in Part I as are also directions for detecting coliform organisms, hemolytic streptococci, tubercle bacilli and *Brucella* organisms in dairy products.

1. FROZEN DESSERTS

A. SAMPLES AND SAMPLING PROCEDURES

Authorized control officials shall take representative samples¹ of frozen desserts at sufficiently frequent intervals to assure consistent conformity of a supply to regulatory standards. Because the quality of the products may vary, take samples at monthly intervals, or more frequently if possible. If necessary to establish complete representation, take multiple and varietal samples. After taking the sample, avoid unnecessary delay before submitting it to the laboratory and before its subsequent bacteriological examination. Wherever possible, maintain samples in a frozen condition.

To determine the conformity of new supplies of frozen desserts to sanitary regulations, examine by the agar plate method not less than 4 samples, each taken on different days from different batches of mix after freezing.

If the sample is to be used for other purposes in addition to a determination of its bacterial content, procure a sufficient quantity to allow for the additional analyses. First, remove aseptically from the sample container the portions required for the bacteriological analyses and reserve the remainder for the other tests. If subdivision of the sample is required prior to the removal of the portions for bacteriological examination, avoid contamination of those portions of the sample.

I. APPARATUS AND MATERIALS

1. AGITATOR

Thoroughly and vigorously stir each batch of unfrozen or melted mix (or melted frozen dessert) immediately before removal of any portion to be submitted for analysis. Wherever practical use a sterile metal agitator, either the disc-attached-to-end-of-rod type, or that with shaft-through-the-bottom (Part I, K, V, 2, p. 139).

2. SAMPLING INSTRUCTIONS

For sampling unfrozen products, preferably use metal tubes (Part I, A, III, pp. 8-9). Sterile glass tubes of similar dimensions have

been used satisfactorily by some workers. Metal cups, 50 ml. capacity preferred, attached to long handles may be used providing they have been subjected to practical sterilization before each use.

For taking samples of frozen mixes, use a clean, dry, sterile knife, spoon, butter trier, wooden spatula or other similar instrument. Completely protect such utensils by wrapping them in so-called kraft paper which does not char easily, or place them with handles outward in containers of glass, metal, or paper provided with suitable covers and sterilize them, preferably in a hot-air oven. Some persons prefer to sterilize the instruments in the field immediately before each use (1) by dipping them in alcohol and then burning off the alcohol, or (2) by exposing them to the flame of an alcohol lamp. These options are permitted provided the instruments are clean and are exposed to sufficient heat to insure sterility.

3. SAMPLE CONTAINERS

Provide clean, dry, sterile, wide-mouthed, screw-top vials, glass-stoppered bottles, clamp-type fruit jars, or other leak-proof containers, preferred size not less than 2 oz.² The use of new liners placed in screw caps each time they are used reduces the chances of leaky closures. Vials and bottles with tops molded or smoothly ground and caps of proper skirt length to obtain leak-proof closures are essential. The use of wide-mouthed, glass-stoppered bottles is limited to conditions where there is assurance of no seepage of refrigerating medium into the container.

4. SHIPPING CASES AND REFRIGERANTS

Wherever possible, use shipping cases equipped to keep samples in a frozen state until ready for analysis, preferably cases in which the refrigerant is frozen brine-pads or dry ice.

A good shipping case consists of an insulated outside container, with a carrying strap, covered with canvas and provided with two brine-pads, one to be placed above and the other below the samples when shipment is made. Two sizes of shipping cases are available, one for 25 and one for 50 2-oz. sample jars. Additional brine-pads may be needed when the larger sized container is used. When brine-pads^{3, 4} are used, they should be allowed to freeze in hardening rooms at an ice cream plant immediately before use. Brine-pads

consist of disc-shaped metal containers approximately 9 in. in diameter and $1\frac{1}{2}$ in. thick, filled with brine. In many places where dry ice is available, corrugated cartons of suitable size with special felt (felt pressed and cemented between two layers of paper) insulating pads can be purchased for protecting frozen desserts during shipment. Corrugated liners may be substituted for felt insulating pads. Put an adequate amount of dry ice not less than 2 in. thick into two large paper sacks (No. 10 size or larger). Place one sack of dry ice in the bottom of the box, and add the separately wrapped containers of frozen dessert. Finally place the second sack of dry ice over the samples before sealing the package.

Partially liquefied mixes and frozen desserts should be frozen before shipment, especially where the interval between taking the sample and making the analysis exceeds 4 hours. When the interval is less than 4 hours, the sample may be packed in water containing cracked ice, providing enough ice is used to maintain the packing medium at a temperature of 40° F. or less, and providing the refrigerant does not contaminate the samples.

When samples are in screw-top vials or glass-stoppered bottles and the refrigerant is water containing cracked ice, the vials or bottles may be dry packed one tier deep in water-tight, metal containers. Then for further protection during transportation, cover these water-tight containers completely with cracked ice in larger, well constructed boxes of wood, metal-lined wood, or metal. To insure proper handling of such shipping cases during transportation and to encourage shipment with bottles in an upright position, attach handles to the cases and label the cases with the words "THIS SIDE UP" on the appropriate surface.

II. STERILIZATION

Sterilize equipment and materials as directed in Part I, A, I, pp. 7-8.

III. COLLECTION OF SAMPLES

To determine the sanitary quality of products at successive stages in their preparation for market, take, in the manner hereinafter described, not less than 50 ml. amounts of unfrozen, partially frozen, or frozen mixes at any time between the preparation of the mix and its sale to the consumer as a frozen dessert.

Immediately after taking the sample, in the presence of the vendor, owner, or manager in charge of the business, identify the container legibly and indelibly with an official number or tag corresponding to the inspection record. Copy all identification marks appearing on the container onto the inspection record. An official seal may be applied to the sample container or to the immediate protective wrapping thereof in such a manner as to assure the recipient at the laboratory that the contents thereof have not been tampered with if the seal is still unbroken when the package is received. Such seals are particularly useful when the inspector desires to leave the samples in hardening rooms to freeze them before shipment to a distant laboratory. The inspection record may be signed by the person from whom the sample was taken, certifying that the sample was taken in a proper manner. Keep a record of the time when the sample was (1) taken, (2) shipped, (3) received at the laboratory, (4) plated, and (5) when the plates were counted.

Rush all samples to the laboratory for prompt examination as hereinafter required. When the time interval between taking the sample and making the laboratory examination exceeds 4 hours, prepare (1) to keep such samples in a frozen condition until tested, and (2) to freeze all unfrozen samples of mix or of melted frozen dessert before shipment or delivery to the laboratory. The preparation shall be such that all samples not only will be received at the laboratory in a frozen condition but also can be maintained frozen until ready for melting preparatory to testing. Samples received at the laboratory too late in the day for testing shall be held in a frozen state until ready for bacteriological examination.

Samples taken locally to be tested promptly—testing in no case to be delayed more than 4 hours—may be packed in water and cracked ice (approximately 40° F.) providing refrigeration at lower temperatures is not available.

1. SAMPLES IN RETAIL SIZED PACKAGES

When sampling retail sized packages of frozen desserts (usually 1 quart or less), obtain a representative percentage of each size, style, and flavor or variety in the unopened package, or transfer aseptically a representative (not less than 50 ml.) portion of each sample to a sterile, leak-proof container. Samples should be of sufficient size to allow for all necessary examinations. If frozen

dessert samples are submitted in their original containers, keep the entire contents frozen until ready to transfer the 50 ml. portion to a sterile, leak-proof container at the laboratory. Protect the caps, covers, and openings of containers from contamination at all times by wrapping each package separately in clean, water-proofed paper or other impervious covering that will give similar protection.

Representative portions of specialties, novelties, decorative and neopolitan types of frozen desserts, (1) may be composited proportionately and the mixture examined, or (2) representative portions of each flavor may be examined separately provided the article is received at the laboratory in a frozen condition permitting satisfactory separation of the several portions. Some workers prefer first to examine a representative composite portion, and then, if the bacterial content is excessive, to examine separately the different component portions of another sample from the same source.

2. BULK SAMPLES

Bulk samples of frozen desserts are examined (1) to determine their bacterial content before or immediately after delivery to the retailer, or as desired at any time thereafter, or (2) to determine the care exercised by the retailer following delivery. The same sampling procedures are applicable when the manufacturer is also the retailer.

For determining the bacterial content of frozen desserts as delivered, sample such bulk lots from either filled or partially emptied containers. After first removing the top inch of frozen dessert with a sterile instrument, collect the sample (not less than 50 ml.) aseptically, with a second sterile instrument, taking the sample from the newly exposed area and transferring it to a sterile container (Part II, A, I, 3, p. 144).

Before determining the bacterial content of frozen desserts as retailed to consumers, or as about to be sold to them, instead of removing the surface portion of the bulk material as specified for delivery samples, take a sample which includes surface portions only to the depth of about $\frac{1}{2}$ in. While the quality of the delivery sample is indicative of the general quality of the product originating in a manufacturing plant, and while the results of the examination may be used to control a large volume of bulk goods, the need for taking retail samples is of equal if not of greater importance, since the results of the examination of the retail sample may be used as a

direct means of control over careless merchants and their vending operations.⁵

Sample individual servings of frozen desserts as dipped by the retailer by taking a representative (not less than 50 ml.) portion with the service dipper or other dispensing device and treat such portion as a regular bulk sample.^{6, 7}

Take samples of unfrozen or partially frozen mixes from the homogenizer or freezer while it is in operation by passing a sterile sampling bottle or cup under the opening at intervals during the discharge.

Sample any mixes as directed in Part I, A, IV, V, pp. 9-10. Agitate thoroughly any mixes that are in cans. If a sampling tube is used, exercise precautions as directed.

If the mix is in a vat, thoroughly agitate the contents of the vat and remove representative portions from at least 6 places as widely separated as possible. Take each sample by inserting a sampling tube slowly with the top of the tube open.

Samples may be removed from cans or vats (1) by dipping a sterile metal cup (approximately 50 ml. capacity preferred) into the unfrozen mix, or (2) by catching the sample of mix in a sterile cup as the mix is poured from the can or vat, provided satisfactory facilities are available for stirring the mix thoroughly immediately before obtaining the sample. Either use transfer containers with long metal handles or hold the container with long-handled, metal clamps. When introducing the sample, handle the sterilized sample containers and the cups aseptically. Subject containers and clamps to practical sterilization between samplings.

Where a number of samples must be taken and only a few sampling tubes are available, a satisfactory procedure for stirring and sampling is described in Part I, A, IV, p. 9.

IV. TRANSPORTATION AND STORAGE OF SAMPLES

When shipping and transporting samples of frozen desserts, use shipping cases or other large, well constructed boxes that will permit the use of sufficient refrigerant to maintain the desired temperatures until the samples arrive at their destination and are ready for examination. When samples are well protected, shipment may be made by express (preferred) or by parcel post, using either dry ice or frozen brine-pads as the refrigerant. The transportation of

samples in a frozen condition packed in dry ice or with frozen brine-pads insures greater uniformity. Parcel post or express shipments of samples in glass-stoppered bottles are apt to be unsatisfactory even when the tops are protected by tightly fitted metal or metal foil or by viscose or parchment paper caps. Where glass-stoppered bottles are used, it is preferable to transport the samples directly to the laboratory under personal supervision to avoid any rise of the refrigerant above the shoulders of the bottles and to keep the bottles in a fixed vertical position to prevent contamination of the contents. When the refrigerant is water containing cracked ice, use the additional precautions given in Part I, A, VII, p. 12.

Do not let the temperature of any sample exceed 40° F. during transportation and before preparation for analysis; if possible, keep all samples frozen until ready for examination. (If dry ice is used, allow for expansion of materials within the container after freezing.)

B. AGAR PLATE METHOD

The value of the agar plate method (Part I, B, pp. 14-37) as applied to the microbiological examination of frozen desserts is attested by numerous references, a few of which are selected,⁸ and records of control laboratories give further evidence of the recognition⁹ of the method. The agar plate method is especially adapted not only to the examination of samples of frozen dessert containing very few bacteria but also to the examination of samples taken at successive stages of manufacture to detect sources of slight contamination.

When the presence of bacteria that fail to grow by the agar plate method is suspected, check the accuracy of the plate count either with the direct microscopic method (Part I, C, pp. 40-57) or by incubation of additional plates at suitable temperatures (Part I, B, VI, 2, p. 30).

On pasteurized products, the plate count may be supplemented by the direct microscopic method for indicating the presence of such types as thermophiles, streptococci, etc. The efficiency of pasteurization and any recontamination after pasteurization may be determined by testing for organisms of the coliform (*Escherichia-Aerobacter*) group (Part I, F, pp. 74-85).

I. SOURCES OF ERROR

The sources of errors when making plate counts of frozen desserts are essentially identical with those occurring when plating samples of milk and cream (Part I, B, I, 1, p. 15).

II. APPARATUS AND MATERIALS

1. WEIGHING BOAT

Provide sterile weighing boats suitable for weighing 1 gm. portions of the frozen dessert.² Use a test tube approximately 5 in. long and $\frac{5}{8}$ in. in diameter plugged with cotton (or other suitable container) in which to sterilize and store each boat and to handle it when weighing the 1 gm. portion (Figure XXX). Transfer the 1 gm. (liquid or melted) portion with a sterile pipette to the weighing boat or dilution bottle.

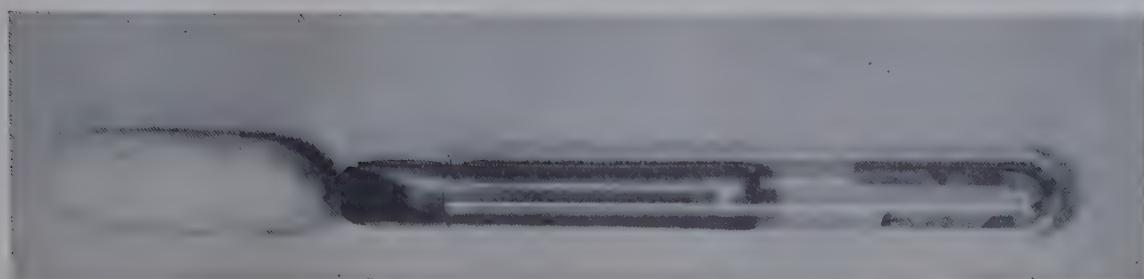


FIGURE XXX—Culture tube with butter-boat as used by the California State Department of Agriculture in their official ice cream control work

2. BALANCE

Provide a balance, 0.03 gm. sensitivity, for weighing the frozen dessert. Periodically, check the accuracy of the weights and the sensitivity of the balance.

3. PIPETTES, GLASSWARE, MEDIA, MEDIA-MAKING EQUIPMENT, INCUBATORS

Use pipettes, dilution bottles, Petri dishes, hot-air ovens, autoclaves, thermometers, incubators and/or incubator rooms, water, beef extract, tryptone, agar, sugars and other chemicals, equipment for determining the hydrogen ion concentration and for preparing media, in accordance with the directions in Part I, B, II, pp. 16–22.

Use standard nutrient agar prepared, adjusted, and sterilized according to the directions in Part I, B, III, pp. 22–25.

III. TEMPERATURE OF SAMPLE

The standard plate count of any sample the temperature of which has exceeded 45° F. for a period of 4 hours is apt to be an unreliable measure of the bacterial content of the frozen dessert when it was sampled.

Do not insert a thermometer into a sample of frozen dessert before the portions for bacteriological examination have been removed. When it is desired to determine the temperature of a sample before removal of these portions, either after storage at low temperatures or when cooling or heating the sample to a desired temperature, insert the thermometer in a similar liquid held in a sample container which has been subjected to treatment identical with that of the sample in question. Allow the thermometer to remain until its reading becomes constant.

Temperatures of melted samples may be determined after the

removal of the portion for analysis. Shaking and handling prior to the removal may increase the temperature slightly, especially if the original sample is small or has been allowed to remain in the heating medium longer than is required just to melt the sample. If there are wide differences in the character of the samples as received, determine and record the temperatures of each lot of samples. When samples are received frozen and maintained in a frozen state until analyzed, a record of this fact is sufficient.

IV. DILUTIONS

When preparing dilutions, observe the precautions in Part I, B, IV, pp. 25-28.

1. MELTING AND AGITATING SAMPLE

Before opening the sample container, remove from the outside of the container all material which may contaminate the contents.

If the frozen dessert at the time of analysis is still firmly frozen, expose it to room temperature for a period not to exceed 15 minutes to prevent delay and unnecessary exposure to subsequent melting temperatures. If the sample is in the original retail sized package, remove aseptically a representative (not less than 50 ml.) portion to a standard 2 oz. (or larger) sterile sample bottle. Immediately before weighing out the test portion, liquefy the sample by placing the container in a water bath at 42°-45° C. (108°-113° F.) *just long enough for the contents to melt*, protecting the contents from contamination by the tempering medium. At no time shall the temperature exceed 45° C. The time interval depends upon the size of both the sample and its container. It is usually 5 to 10 minutes, and must not exceed 15 minutes. These requirements may be met (1) by using not too large a sample, (2) by providing sufficient tempering medium, and (3) by maintaining the temperature as desired.¹⁰ Rotate containers during the melting process to reduce the melting period. When the frozen dessert has just melted, immediately before measuring out the desired quantity, shake the sample container not less than 25 times (as required in Part I, B, IV, 3, p. 26) to insure uniform distribution of the contents. In a very few samples excessive foaming may occur when the sample is shaken.⁴

Because of the greater time interval required when preparing initial dilutions of frozen desserts over that required for pipetting milk and cream samples, it is essential that the number of samples set up at a time be limited to approximately 8 so that the time interval of exposure of the mixtures to high temperatures will be reduced uniformly to not more than 15 minutes.¹¹

Where pieces of nuts, fruits, seeds, and the like, interfere with representative sampling for bacteriological analyses, some workers prefer to examine the liquid portion only after mechanical separation of the solid pieces has been accomplished under aseptic conditions. This practice is not recommended because of the inevitable added delay, exposure to contamination, and actual loss of identity of the original sample. When the original sample has been found to have a high bacterial content, the examination of additional samples may possibly be of greater value than an examination of the separated, liquid portion. Furthermore, the bacteria are probably not in the nut and seed portions and they are less apt to be in the fruit and confectionery portions of the mix than in the liquid portions. When larger quantities are used in the initial dilutions of those frozen desserts containing pieces of fruits, nuts, seeds, etc., the need for the separation of the solid portions from the liquid portions is less apparent. In cases where the sample is not uniform, an average of the counts from several portions of the same sample is preferable to a count from a single analysis made after the solid materials have been separated from the liquid portion. The process of separation inevitably introduces errors due to evaporation and to mechanical losses and thus causes errors in the calculation of the results on the basis of the original sample.

2. MEASURING THE SAMPLE

Use a sterile pipette and weigh aseptically into a sterile butter boat * a 1 gm. (or larger) representative portion of frozen dessert or mix; or weigh 11 gm. of the material directly into a dilution bottle, ^{2, 3, 12} which may or may not contain 99 ml. of sterile water when the frozen dessert is measured into it. The use of an 11 gm. (or 11 ml.) portion in 99 ml. water gives a 1:10 initial dilution.

If a culture tube is used to contain the butter boat (Figure

* Made by Mojonner Bros. Co., Chicago, Ill.

XXX) when weighing the sample, place the culture tube on the pan of a sensitive balance (Part II, B, II, p. 150), having adjusted the boat so that it extends $\frac{3}{4}$ in. out of the end of the tube. After weighing the desired amount of sample accurately to the second decimal place, slide the butter boat with its contents out of the tube into a dilution bottle containing 99 ml. sterile water. Dilution bottles should have large enough neck openings to admit the butter boat freely.

The practice of sterilizing several butter boats in a larger container, such as a pint jar, and of removing the individual boats aseptically to a specially modified torsion balance pan for weighing the frozen dessert has been found satisfactory if the weighings are made in a room protected from drafts and dust. Two parallel metal strips about $\frac{3}{8}$ in. high and $1\frac{1}{2}$ in. long, fixed vertically and spaced so as to keep the boat from rolling off or touching the pan of the balance make a satisfactorily modified balance pan. Other forms of modified pans (or cradles) when detachable from the balance and enclosed in wrapping paper or otherwise protected for sterilization in an oven, would be satisfactory. When placed on the balance these can be used repeatedly for weighing samples. Where there is doubt as to the continued sterility of the support, several sterile ones may be provided for intermittent use. Such aseptic precautions as sterilizing the forceps used for handling the individual boats are obviously necessary. Protective shields above the balance pan may be employed where necessary. The advantage of this method lies in the convenience of balancing each boat singly on a $1\frac{1}{2}$ in. balance pan as against balancing a test tube holding a partially extended boat. Butter boats may be nearly adjusted to the desired weight by grinding or clipping portions from overweight boats or by soldering portions to those that are underweight.⁴

Because of overrun, viscosity of the material, and differences in densities depending upon varying compositions and viscosities at different temperatures, do not use volumetric measurement of frozen desserts unless no means can be made available for gravimetric measurements.¹³ The measurement of frozen dessert samples with a pipette is apt to be unsatisfactory and should not be used where punitive measures are involved. Volumetric measurements usually require that the time for heating the frozen dessert be longer than that required prior to gravimetric measurements; this is to provide

minimum viscosities at the limiting temperature for pipetting the liquid.

If the volumetric method is used, preferably transfer 11 ml. of the sample to a dilution bottle containing 99 ml. sterile water. Insert the pipette slightly beneath the foam layer when removing the portion for analysis. After shaking vigorously, prepare a 1 : 100 dilution by transferring 11 ml. to a second dilution bottle containing 99 ml. of sterile water.

In the examination of finished frozen desserts, if a pipette is used, withdraw preferably at 20° C. (68° F.) a sufficient quantity of frozen dessert to assure the delivery of the desired amount, well mixed but as free as possible from air bubbles. Let the column drain to the apparent rest point of the liquid in the tip of a standard A.P.H.A. 1.0 ml. pipette, or similar pipette, and blow out the last drop as quickly as possible without touching the pipette to the dilution bottle. Ordinarily the entire operation including the drainage and complete deposition requires 2-3 seconds. If an 11 ml. standard A.P.H.A. pipette is used, the required interval is 3-4 seconds. During transfer to the bottle do not let any liquid from the outside of the pipette mix with the measured contents of the pipette or fall accidentally into the dilution water. It is essential that each laboratory actually predetermine the manner of use by trial and error measurements (weighings of the amounts delivered by the pipette) to establish the quantity of melted mix that will be required for the exact measurement of the desired amount of the sample under the widely varying conditions of viscosities and densities.

In examining samples of frozen dessert mixes, do not wipe or drag the pipette across the lip and neck of the sample containers or dilution bottles when the original quantity of mix or its subsequent dilutions are removed. Make transfers carefully because each drop of mix or of dilution water falling into the bottle or on the plate from the outside of the pipette may be expected to increase the count of the next higher dilution by at least 5 per cent. When measuring dilutions, hold the end of the pipette at an angle of 45° against the Petri dish, the neck of the dilution bottle or the rod extending through the stopper into the dilution bottle; allow the diluted mix to drain from the graduation mark to the apparent rest point in the tip of the pipette, and then touch only once against a

dry spot on the glass. Ordinarily, the drainage and complete deposition requires 2-3 seconds. Use care to raise the Petri dish covers only enough to insert the pipette.

V. PLATING, INCUBATING, COUNTING PLATES AND RECORDING COUNTS

When plating frozen dessert samples, when incubating and counting the plates, and when recording the counts, observe the precautions stated in Part I, B, V-VIII, pp. 28-37. The time interval between making the first dilution for any series of samples and pouring the first plate with agar should not exceed 20 minutes. Unless otherwise specified, the count shall be reported as the Standard Plate Count. Use the same temperature of incubation that is used for routine market milk work. Enter the temperature of incubation on all record blanks.

VI. INTERPRETATION OF COUNTS

When interpreting counts use the precautions in Part I, B, VIII, pp. 35-38. High standard plate counts in frozen desserts may result from one or more of the following causes¹⁴:

1. Poor quality of ingredients such as cream, milk, gelatin, sugar and other products,¹⁵
2. Improper processing such as inefficient pasteurization or aging too long at too high a temperature,¹⁶
3. Inadequate refrigeration of ingredients, of mix before pasteurization or of mix after pasteurization,
4. Improper cleaning and sterilizing of equipment such as storage vats, homogenizer, pasteurizer, aging vats, pipe lines, cans, etc.,¹⁷
5. Careless employees who either knowingly or through ignorance neglect to process the mix properly or to clean carefully the equipment used in its manufacture.¹⁸

A high standard plate count obtained on a frozen dessert, therefore, usually does not indicate an unsafe product but more properly should be taken to indicate a neglected product. The standard plate count of a frozen dessert, as of other dairy products, has been used for many years as an index of general sanitation. Frozen desserts having a high standard plate count do not necessarily contain disease producing bacteria, and those having a low plate count are not necessarily free from them.¹⁹ Therefore, combine sanitary inspec-

tions with laboratory analyses before basing conclusions solely on the latter.

The sterility of equipment may be determined as described in Part I, K, pp. 125-127. The microbiological character of the ingredients may be determined using methods described in Part II, 2, pp. 162-211.

VII. REPORTING COUNTS

Follow in general the methods of reporting counts that are given in Part I, B, VIII, pp. 35-38.

C. DIRECT MICROSCOPIC METHOD

The direct microscopic method for estimating bacterial populations in frozen desserts consists (1) of examining under the oil immersion lens of the compound microscope specially prepared and stained films of frozen desserts which have been dried on glass slides, or (2) of counting either the individual bacteria or the groups of bacteria in limited areas of the films so prepared. The general bacteriological condition of the frozen dessert may be determined after a rapid examination of a few microscopic fields. An actual count will give a quantitative estimation of the number of cells or groups of cells (individual unclumped cells counted as a group) present and the counts so obtained are reported respectively as individual microscopic counts and group microscopic counts.

The direct microscopic method has been applied successfully to the examination of frozen desserts to determine both the total numbers and, to a limited degree, the types of bacteria present.²⁰ The microscopic method offers a rapid routine technic because within 15 minutes after a film has been prepared, the results of the examination may be made available. The presence of large numbers of bacteria is readily detected on the films, whereas the absence of noticeable numbers of bacteria confirms the accuracy of low standard plate counts by the agar plate method. While the consensus of opinion is that living bacteria take the stain deeply and that very faintly stained organisms are dead, the presence of large numbers of cells on the slide, regardless of whether they are living or dead, indicates a faulty condition.

Either of two kinds of counts or estimates may be made by the microscopic method: (1) a count of the individual bacteria seen, and (2) a clump count including both the isolated bacteria and the undivided groups, each clump being counted as one organism. Since each unseparated clump of bacteria is presumably capable of growing into a colony, the clump count in a general way approximates the standard plate count, described in Part I, A, I-VIII, 14-37, but no attempt should be made to reinterpret plate counts in terms of microscopic counts or to convert microscopic counts into plate counts (Part I, C, I, 4, pp. 42-43).

I. APPARATUS AND MATERIALS

When collecting samples observe the precautions and use the standard apparatus outlined and described in Part I, C, II, 1-5, pp. 44-49.

II. ADJUSTMENT OF MICROSCOPE

Carefully adjust the microscope, following the instructions given in Part I, C, III, 1-2, pp. 49-50.

III. TEMPERATURE OF SAMPLE

For discussion of temperatures at which samples shall be maintained during collection and until examined and for directions for taking temperatures of samples, see Part II, 1, B, III, p. 151 of this Report.

IV. PREPARING THE FILM

When making the films exercise the precautions discussed in Part I, C, IV, 1-4, pp. 51-53. Prepare smears by spreading 0.01 ml. melted mix or melted frozen dessert over a 1 sq. cm. area in the usual manner. Dry the smears on a warm plate, drying them a second time after a second treatment with xylene and alcohol if fat drops appear after the first drying or there is any tendency for the preparation to separate from the slide. Avoid drawing air bubbles into the pipette.

The Fay method²¹ of preparing films may be used but is not recommended as excellent 1 sq. cm. films can be prepared if directions are followed. Particles of chocolate, cocoa, nuts or fruits in a frozen dessert interfere with the uniform distribution of the materials on the slide. If the frozen dessert is free from such interfering substances and is still too viscous to distribute uniformly on the glass slide, a portion may be diluted with an equal volume of sterile skim milk before making the film.

V. COUNTING THE BACTERIA

Use the same precautions when counting the bacteria in frozen desserts that are necessary when counting them in milk and cream samples (Part I, C, V, pp. 54-55. Frozen desserts of high quality should show less than an average of one bacterium per field. If molds and yeasts are present in large numbers they will be evident in the microscopic preparations.

D. COLIFORM ORGANISMS IN FROZEN DESSERTS

Use the method outlined in Part I, F, pp. 74-85, when determining the coliform (*Escherichia-Aerobacter*) content of frozen desserts. Exercise due caution when interpreting the results of this test because these organisms are less readily killed at pasteurizing temperatures in foods rich in sugars and added butter fat than when present in market milk.²² Obviously, higher pasteurization temperatures can be used on frozen desserts than on market milk and cream without injuring their quality.

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2. INGREDIENTS OF FROZEN DESSERTS

A. MICROBIOLOGICAL EXAMINATION OF EVAPORATED MILK (STERILIZED) USED IN FROZEN DESSERTS

Evaporated milk as found upon the market is primarily confined to cans in which the product supposedly has been subjected to sterilization by heat. The bacterial count of such milk should be zero; if not, it usually is relatively low and for that reason the bacterial analysis is somewhat different from that of other related products.

Use the standard agar plate method as for fluid milk, Part I, B, I-VIII, pp. 14-37, for the examination of evaporated milk with certain modifications as hereinafter noted.

I. SAMPLES: COLLECTION AND TRANSPORTATION

As evaporated milk is found mainly in sealed, sterile containers the collection of samples usually consists in taking the original containers of the product to the laboratory. Because of the ordinarily sterile condition of the product, refrigeration is not regarded as necessary while the unopened containers are being transported to the place of examination. All containers shall be marked, and proper records made regarding the sample.

II. APPARATUS AND MATERIALS

The apparatus and materials used shall conform in all respects to Part I, B, II, pp. 16-22, and should be sterilized in accordance with Part I, A, I, pp. 7-8, and B, II, 4, p. 19.

III. PREPARING THE SAMPLE AND MAKING DILUTIONS

It is very important that no contamination shall occur when obtaining the sample from the original container for dilution. All utensils that are used or in any way come in contact with the product must be sterile and the room in which the work is carried out shall be as free as possible from dust contamination.

The approved method of handling the container previous to sampling is to shake the can and its contents thoroughly, remove paper labels, wash the outside of the can with warm water, wipe

dry with a clean towel, sponge with 5 per cent phenol, dip the surface to be punctured in alcohol, and flame. In cases where it is desired to seal the can after removing the sample, the surface shall be coated with solder before being punctured. This procedure will materially aid in the sealing process. The can opening appliance shall be sterilized in a flame before using. The opening shall be only large enough to permit the insertion of a sterile (usually 1 ml.) pipette. Remove a suitable quantity of the contents with a pipette as rapidly as possible. The opening shall be protected for a short time by covering the entire can with one-half of a sterile Petri plate. If further work is to be carried out with the sample, reseal the can or transfer the contents to a sterile, closed bottle and store at refrigeration temperature.

If the sample of evaporated milk completely fills the container in which it is received, transfer the entire contents to another clean, dry, sterile container of ample size to permit proper mixing by shaking.

For direct plating of a low count product, distribute 1 gm. portions of the sample directly into each of 5 Petri plates. For 1:10 dilution, measure 11 gm. of evaporated milk directly into a dilution bottle containing .99 ml. of sterile, distilled water. If higher dilutions are desired, prepare them from the 1:10 dilution. See also Part I, B, IV, 1-6, pp. 25-28 for further details.

In the case of abnormal, coagulated samples (which should not be used for food purposes) the use of tenth-normal lithium hydroxide (N/10 LiOH)¹ as diluent is recommended. This diluent facilitates solution of such samples. Keep the diluted product thoroughly agitated prior to the withdrawal of the sample with the pipette in order that a representative sample of the solution may be transferred to the Petri plate.

IV. PLATING MEDIUM

Use standard nutrient agar prepared according to Part I, B, III, 1-3, pp. 22-25.

V. PLATING

Follow the directions outlined in Part I, B, V, p. 28 in pouring the plates. With normal evaporated milk, direct plating of the product (1 gm.) should yield either no colonies or, at most, very few in the 5 plates. Plate the usual dilutions in every case, with two sets of duplicate plates for each dilution, one for incubation

at 37° C., and the other at 55° C. (For plates incubated at 55° C. use 15–18 ml. of standard nutrient agar.)

VI. INCUBATION

In incubating plates made from evaporated milk, use the same incubation temperatures that are used for routine milk and cream work (Part I, B, VI, 1 and 2, pp. 29–31).

VII. COUNTING PLATES

The procedure for counting plates shall follow that outlined in Part I, B, VII, 1–6, pp. 31–35, except that the plan of counting only those plates showing 30–300 colonies cannot be followed due to the small number of colonies found in the normal product. In the case of abnormal samples or those containing material which fails to go into suspension readily, exercise care in counting the plates to differentiate between bacterial colonies and undispersed masses of the constituents of the sample. Doubtful objects shall be examined with sufficient magnification to determine whether they are colonies.

VIII. REPORTS

A plate count on a normal evaporated milk shall be reported as the Standard Plate Count per milliliter except where 1 gm. has been plated on each of 5 plates. Where the direct plating of 1 gm. portions into each of 5 plates has been used the colony count will be the total count of the 5 plates, and shall be expressed as the Standard Plate Count per 5 gm. For further details see Part I, B, VIII, pp. 35–37. Enter the incubation temperature used on all record sheets.

IX. TEST FOR STERILITY

For determining whether evaporated milk in a can is sterile, hold sample cans at 37° C., and at 55° C., for 1 week before plating contents. Cans in which an abnormal physical condition of the milk has developed may have large numbers of organisms present, so that high dilutions may be necessary in plating such samples. Cans which show milk in normal condition and which yield no colonies on any of the plates, including 1 ml. direct plating, shall be assumed to be sterile.

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B. MICROBIOLOGICAL EXAMINATION OF CONDENSED MILK (INCOMPLETELY STERILIZED) USED IN FROZEN DESSERTS

Under this heading are included those products that may or may not contain added sugar but which supposedly have not been sterilized in the container. Sweetened condensed milk is commonly sold at retail in hermetically sealed cans. However, a great deal of the sweetened and the unsweetened product is handled in bulk containers, such as 10 gallon cans, barrels, tank trucks, or tank cars.

Use the standard agar plate method, Part I, B, I-VIII, pp. 14-37 as for fluid milk for the examination of condensed milks with certain modifications as hereinafter noted.

I. SAMPLES: COLLECTION AND TRANSPORTATION

Follow the procedure outlined for evaporated milk (Part II, 2, A, I, p. 162) in sampling products enclosed in hermetically sealed cans. If the sweetened condensed milk is very thick and viscous, place the canned product in a water bath at a temperature not over 37° C. for a short time so that the product will become less viscous. If the separation of sugar has been unusually great, transfer the entire contents of the container, including sediment or other deposits, to a sterile receptacle to insure proper mixing.

When samples are taken from barrels or other large containers precautions are necessary to remove the samples aseptically. Also, it will be difficult to mix the contents thoroughly. Do not overlook the danger of contaminating the sample when it is being drawn from a storage tank. The movement of valves or pipe joints may dislodge large numbers of organisms that may contaminate the sample and give misleading results. If samples must be taken from large volumes of the product, obtain them by using sterile tubes, dippers, or other suitable equipment.

If a sample representative of the product which will go into frozen desserts or other food products is desired, do not measure the top layer but insert the sampling tube so as to obtain a cross-section of the entire product. If samples are to be taken for research

work or for other microbiological studies where it is desired to exclude surface contamination, then the top 3-4 in. may be removed with a sterile instrument before the sample is taken. Where it is necessary to take composite samples, use a container of sufficient size to allow for mixing the final sample.

If the sample of condensed milk completely fills the container in which it has been collected or is received, transfer the entire contents including sediment or other deposits, to a second clean, dry, sterile container, using the precautions that should be applied in any sampling for a microbiological analysis.

In the case of hermetically sealed cans of sweetened condensed milk the temperature of storage and transportation of samples is not important because the product usually does not deteriorate at ordinary temperatures.

In the case of samples from bulk condensed milk, the samples shall be kept under refrigeration as outlined in Part I, A, VI, pp. 11-12.

II. APPARATUS AND MATERIALS

The apparatus and materials should conform in all respects to the requirements of Part I, B, II, pp. 16-22, and be sterilized in accordance with Part I, A, I, pp. 7-8 and B, II, 4, p. 19.

III. PREPARING SAMPLE AND MAKING DILUTIONS

For both plain unsweetened condensed milk and sweetened condensed milk the gravimetric method shall be used for preparing dilutions. The dilutions shall be prepared either by weighing 11 gm. condensed milk directly into a bottle before the addition of the dilution water or, if desired, the milk may be weighed into the bottle containing the dilution water.

In the case of abnormal, thickened milk, use sterile pieces of broken glass or sterile glass beads in the dilution bottle to assist in the mixing of the sample in the dilution water. In such products, tenth-normal lithium hydroxide ($N/10$ LiOH)¹ as a diluent is recommended as an aid in dissolving the coagulated material.

IV. PLATING MEDIUM

Standard nutrient agar prepared according to Part I, B, III, 1-3, pp. 22-25, shall be used.

V. PLATING

Follow the directions outlined in Part I, B, V, p. 28, in pouring plates. It is ordinarily possible to count plates with 30–300 colonies with these products.

VI. INCUBATION

In incubating plates made from condensed milk, use the incubation temperature that is used for routine milk and cream work (Part I, B, VI, 1 and 2, pp. 29–31). Enter the incubation temperature used on all record sheets.

VII. COUNTING PLATES

Follow the procedure for counting plates as given in Part I, B, VII, 1–6, pp. 31–35.

VIII. REPORTS

Report results as Standard Plate Count per gram of product. Because few bacteria are ordinarily present in condensed milk some plates may show less than 30 colonies; accordingly, the actual number of colonies shall be used in calculating the count which is to be reported as the Standard Plate Count.

IX. TESTS FOR ORGANISMS OF THE COLIFORM GROUP

Handling of condensed milk in bulk offers more chance for contamination than does handling of the canned product. Since members of the coliform (*Escherichia-Aerobacter*) group are practically eliminated by the ordinary process of manufacture, the frequent presence of this group of organisms in plain condensed milk usually indicates that contamination has occurred.

The use of violet red bile agar as outlined in Part I, F, V, 3, p. 79, is suggested for dilutions above 1:10, supplemented by brilliant green lactose peptone bile broth as outlined in Part I, F, V, 1, p. 77 for lower dilutions.

X. YEAST AND MOLD COUNTS

In condensed milk containing sucrose, the determination of yeast and mold counts furnishes additional information. Excessive numbers of yeasts in condensed milk are normally self-evident; the presence of even a few yeasts is a sign of serious contamination. The presence of mold in condensed milk should be considered as an indication of inadequate sanitation.

Yeast and mold counts shall be made as outlined in Part I, J, III, 1-7, pp. 112-113.

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C. MICROBIOLOGICAL EXAMINATION OF DRY MILK USED IN FROZEN DESSERTS

I. SAMPLES: COLLECTION AND TRANSPORTATION

It is important that all utensils and containers used in sampling¹ dry milk be clean, dry, and sterile; also that the manipulations be performed as rapidly as possible since dry milk rapidly absorbs moisture from the air.

Obtain samples from standard bulk packages, such as barrels, drums, or boxes by use of a sampling tube or a device similar to an elongated butter or cheese trier. Such a device may not remove an unbroken column of dry milk, but in general a representative sample obtained through the entire depth of the container can be secured by such a device, the efficiency of which is determined by its design and manipulation. Withdraw at least three plugs or columns of dry milk from each container.

Secure samples from a bulk or mass of dry milk not contained in a standard package from various points throughout the mass of dry milk by means of a sterile tablespoon or wide spatula. Combine such samples in suitable sterile containers from which, after properly mixing, a composite sample may be obtained. To prepare a composite sample from portions obtained as described above, mix the samples in a clean, dry, sterile container of sufficient size to permit thorough mixing by shaking the dry milk so that the sample withdrawn from the mixture will be representative. Since dry milk powders are hygroscopic, this should be done in a dry atmosphere in as short a time as possible.

If the dry milk in contact with the inner surfaces of the containers has become wet or dirty, these portions will probably show much higher bacterial counts. Therefore, separate samples should be collected of this type of material, the results reported separately, and notation made of the condition.

Keep samples in air tight containers after collection until ready for analysis because of the hygroscopic qualities of powdered milk. Analyze samples as soon as possible after they are collected as the bacteria in dry milk tend to die out during storage of the dried product.²

II. APPARATUS AND MATERIALS

Use single or double friction-top cans, previously cleaned and sterilized, for collecting, storing, and shipping samples. Glass jars or bottles equipped with tightly-fitting screw tops are also satisfactory, except that their fragility requires care especially in shipping.

Use sterile tablespoons, wide spatulas, or triers for taking samples from a bulk or mass of dry milk not contained in standard packages.

Use pipettes for making dilutions of reconstituted dry milk that conform to the specifications recommended in Part I, B, II, 1, pp. 16-17. Use dilution bottles and Petri dishes which conform to the specifications in Part I, B, II, 2-3, pp. 17-19.

An aluminum weighing boat or scoop furnished with a counterweight * (Figure XXXI) is recommended for use in weighing the



FIGURE XXXI—Aluminum weighing boat with counterweight

samples. These boats are convenient and satisfactory since they are easily cleaned and may be sterilized by flaming. Their use makes it easy to transfer the weighed samples into the dilution bottles.

* Sargent and Co., Chicago, supply satisfactory boats

The tablespoons, spatulas, triers, etc., may be wrapped in kraft paper or put in metal containers with handles outward, and sterilized in a dry-air sterilizer the same as the glassware; or, if they are to be used immediately they may be sterilized in the laboratory by very thorough flaming, and wrapped in sterile towels to protect them until used. If several samples are to be secured at the same time, the triers, spoons, and spatulas used for sampling may be cleaned and sterilized during sampling operations by wiping them thoroughly with a clean, dry cloth, then by sponging them off with cotton moistened with alcohol, followed by flaming thoroughly with an alcohol lamp or other burner.

Use materials in making microbiological examinations of dry milk which conform to the specifications given in the section on bacteriological examination of fluid milk (Part I, B, II, 8-14, pp. 21-22).

Sterilize glassware, according to the directions in Part I, A, I, pp. 7-8 and B, II, 4, p. 19.

III. PREPARING SAMPLE AND MAKING DILUTIONS

Use the same general precautions in preparing dry milk samples that are used for any samples taken for bacteriological analysis.³ If the sample of dry milk completely fills the container in which it is collected or received, transfer the entire contents to another larger, clean, dry, sterile container of ample size to permit proper mixing. It is important to prevent extraneous contamination during the mixing and handling of the product.

Weigh the sample of dry milk under aseptic precautions into a sterile, aluminum weighing boat or scoop when it is ready for analysis. A torsion balance, 30 mg. sensitivity, is sufficiently accurate for satisfactory weighing. From the thoroughly mixed sample, weigh 11 gm. dry milk and add to 99 ml. sterile water. Use this size of sample as it is more likely to be representative than a smaller one.

It is important to prepare the dilutions so that: (1) the milk fat, if appreciable amounts are present, will not be churned out of suspension, and (2) the poured plates will be as free as possible from undissolved particles of dry milk that may be confused with pin point colonies when the plates are examined after incubation.

Specific directions are given in Part I, B, IV, 3, p. 26 for shaking the dilutions. There is some difficulty in dissolving dry milk powder completely. Shaking must not be too severe or prolonged or it will churn out the milk fat if appreciable quantities are present. Mild agitation followed by a short soaking period (1 to 3 minutes) and then more vigorous shaking frequently facilitates dissolution of the particles. If warmed dilution water² is used to dissolve the samples, greater care must be taken not to churn out any milk fat present.

Spray-dried milks are much more soluble than drum-dried milks; hence it is easier to prepare satisfactory dilutions from them. It is suggested that unless the history of the sample is known it be examined under low magnification to determine the method used in drying it. The drum-dried product will appear as small, irregular flakes and the spray-dried product as globules or clusters of globules. The more soluble dry milks will dissolve with little difficulty in the distilled water dilution blank. If careful warming of the dilution water blanks to 43–49° C. before the weighed sample is added does not dissolve the powder satisfactorily, then the use of an alkaline dilution blank is recommended.^{3, 5}

Lithium hydroxide dilution blanks (containing N/10 LiOH) are recommended⁵ for use with dry milks that do not dissolve readily. Although the pH values of dry milk dilutions are slightly increased when lithium hydroxide blanks are used, the pH values of the inoculum-medium mixtures seeded from lithium hydroxide blanks can be controlled largely by adjusting the reaction of the medium; *i.e.*, use medium with reaction pH 6.6 at the lower end of the permitted range (Part I, B, III, 1, pp. 22–23). The pH can be further controlled by using lithium hydroxide dilution blanks for the original 1:10 dilution only and using sterile, distilled water blanks for higher dilutions. Lithium hydroxide dilution blanks, used as recommended, are not germicidal. The precaution of using chemically clean glassware—especially dilution bottles (Part I, B, II, 2, pp. 17–19)—is important when lithium hydroxide dilution blanks are employed.

IV. PLATING MEDIUM

Use nutrient agar, conforming with the requirements given in Part I, B, III, 1–3, pp. 22–25.

V. PLATING

Follow the precautions and technic given in Part I, B, V, p. 28 for the inoculation of plates from fluid milk samples. Make dilutions which will give neither more than 300 nor less than 30 colonies on the plate. Plate immediately after the dilutions are made and complete the process of pouring agar into the plates within 20 minutes after the time the sample of dry milk is first mixed with the dilution blank.

VI. INCUBATION

In incubating plates made from dry milk, use the same temperature that is used for routine milk and cream work (Part I, B, VI, 1, p. 29).

If the presence of thermophilic bacteria is suspected, pour additional plates and incubate at 55° C. for 24 to 48 hrs.^{4, 7} Pin point colonies are quite likely to develop at this temperature. Exercise caution in order not to confuse them with particles of undissolved milk solids. This incubation temperature is recommended as many dry milks during their manufacturing process are held at temperatures favorable for the development of true thermophilic bacteria. Follow the procedure given in Part I, B, VI, 2, pp. 30–31 closely. Incubate at 18 to 25° C. for 3 to 5 days if the presence of bacteria capable of growing at lower temperatures is suspected.

VII. COUNTING PLATES

The precautions to be employed in counting plates are described in Part I, B, VII, 1–6, pp. 31–35. If there are no plates within the recommended 30–300 colony limits, count the plate that comes the nearest to the upper limit.

Since many samples of dry milk contain appreciable numbers of aerobic spore-formers, plates poured from these samples will frequently contain many large, spreading colonies which make it difficult if not impossible to count the number of colonies accurately. This occurs most frequently on the plates made from the low dilutions. When spreaders do occur in the plates that fall within the recommended limits for counting (30–300 colonies), the best estimate possible shall be made of such plates; this estimate shall be checked by counting the plates of the next higher dilution. The use of porcelain tops on Petri dishes of the type that is glazed on the outer surface will be found useful in making these counts.

An essential precaution to observe in counting plates poured from dry milk samples is not to confuse small pin point colonies with specks or particles of undissolved milk solids. If such confusion does occur, an abnormally high count will be obtained. If the sample is soluble with difficulty such particles are very likely to be present unless alkaline dilution blanks are used. The best way to distinguish between the particles and pin point colonies is by examination under the low power magnification of a microscope, preferably of the Greenough type.

VIII. REPORTS

Report the results as the Standard Plate Count per gram of dry milk. Record the temperature of incubation used. See Part I, B, VIII, pp. 35-37 for further discussion.

IX. MICROSCOPIC COUNTS

Because of the germicidal effect of dehydration and storage, the determination of the number of viable bacteria present in a dried milk does not give an accurate picture of its sanitary history. A direct microscopic examination of a stained preparation of a dry milk is therefore quite valuable, for this procedure gives information practically unobtainable otherwise. With dry milk samples that dissolve easily the procedure described in Part I, C, pp. 40-57 is recommended as the most satisfactory method for using the microscopic technic. Use the 1:10 dilution of the dry milk in distilled water for making the smear.

With samples of dried milk that are soluble with difficulty it is very troublesome, if not impossible, to secure a uniform, finely dispersed suspension in the distilled water blank; as a result, a very uneven film of milk solids is obtained on the slide. Such films are not only frequently washed off during staining, but also the bacteria are often so obscured by clumps of casein that it is impossible to make a satisfactory examination of the smear. Dissolve such samples in lithium hydroxide dilution blanks. When smears made from the 1:10 dilution in N/10 LiOH are to be examined microscopically, the following method⁵ of staining is recommended. Mix 0.01 ml. methylene blue and 0.01 ml. alkalized dry milk dilution on the slide. Spread over an area of 1 sq. cm. Dry on a level surface at room temperature. Immerse in 95 per cent ethyl alcohol for about 1 minute. Dry and examine under an oil immersion

objective. The immersion in alcohol partially decolorizes the background and causes the bacterial cells to stand out more distinctly.

A method⁸ of preparing microscopic preparations of difficultly soluble dry milk follows:

1. Prepare a 1:10 dilution of the dried milk sample in tenth-normal lithium hydroxide (N/10 LiOH).
2. Pipette 0.01 ml. of the 1:10 dilution onto a clean glass slide and spread over an area of 1 sq. cm.
3. Dry smear at 37–40° C.*
4. Immerse smear in xylene for 1–2 minutes. Dry in air.
5. Immerse smear in fixing solution (2 per cent calcium chloride in 95 per cent alcohol, 2 drops glacial acetic acid being added per 100 ml. fixing solution) for 5 minutes.
6. Wash smear in water and dry in air. *Smear must be thoroughly dried before staining.*
7. The fixed smear may be stained according to either of the following methods:
 - a. Use a methylene blue solution that meets the requirements of Part I, C, IV, 4, p. 53.
 - b. Use North's anilin oil methylene blue stain† which is prepared as follows:
 - A. Anilin oil—3 ml.
 - B. 95 per cent ethyl alcohol—10 ml.Mix solution A with solution B. Add 1.5 ml. concentrated hydrochloric acid slowly with constant agitation. Add 30 ml. saturated alcoholic methylene blue solution slowly. Add distilled water to produce a total volume of 55.5 ml. Filter. Stain dried smear for 15 seconds.
8. Wash stained smears by repeated immersion in a beaker of water, then place on a horizontal surface and allow to dry.

Report the results of the microscopic count as the number of individual bacteria per gram of the dry milk. Obviously it is neces-

* Dried milk smears prepared from the 1:10 dilution in N/10 LiOH do not require any fixation other than 95 per cent alcohol when stained with North's anilin oil methylene blue stain. However, when plain methylene blue stain, Gram stain or other stains are applied to dry milk dissolved in LiOH solution the special fixation must be employed.

† This stain is very lightly absorbed by the milk protein in a smear, while the microorganisms in the preparation take a deep blue color, thus facilitating recognition of the organisms. Significantly higher counts have been reported for smears stained with this solution than for duplicate smears stained with plain methylene blue stains.

sary to multiply the counts obtained by microscopic examination of the 1:10 dilution by 10, and the precautions discussed in Part I, C, V, pp. 54-55 also apply.

X. TESTS FOR ORGANISMS OF THE COLIFORM GROUP

When organisms of this group are found in dry milk^{9, 10} their presence has much the same sanitary significance as it has in fluid milk (Part I, F, I-III, pp. 74-76). Tests for the presence of coliform (*Escherichia-Aerobacter*) organisms help to determine the efficacy of the heat treatment that dry milk has undergone much as do tests for coliform organisms in pasteurized milk.¹¹ Use the method of testing for organisms of the coliform group given in Part I, F, pp. 74-85, using the 1:10 dilution of the dry milk sample as the basis for making the inoculations.

XI. TESTS FOR HEMOLYTIC STREPTOCOCCI AND STAPHYLOCOCCI

Due to the increasing prevalence of food poisoning outbreaks caused by hemolytic staphylococci and in some instances by hemolytic streptococci,¹²⁻¹⁹ it is recommended that tests for these organisms be made.

Follow the detailed directions given in Part I, G, I-III, pp. 87-92 for the recognition of hemolytic streptococci. Follow the same procedure for detecting hemolytic staphylococci except that these will of course exhibit their own characteristic morphology. However, for growing staphylococci, sheep or even rabbit blood is preferred to horse blood.

Although not all hemolytic cocci are toxigenic in foods, their presence in dry milk suggests that additional investigation should be made. Tests²⁰⁻²⁴ can be made, if desired, to determine the toxigenic properties of these organisms.

XII. MOLD COUNTS

The mold content³ of a dried milk is partly dependent on the handling and exposure subsequent to drying, and with a spray-dried product the mold content also reflects the efficiency of the air filter of the spray dryer. As the use of lithium hydroxide dilution blanks has little or no effect on mold counts, it is satisfactory to use the dilutions made with these blanks in determining the mold counts of powders soluble with difficulty. Use the method for determining

the mold count of dried milk given in Part I, J, III, pp. 112-113. However, malt agar (the dehydrated form is suggested to obtain greater uniformity) with reaction of pH 4.5 may also be used.

Results shall be reported as Mold Plate Count per gram of dry milk.

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D. MICROBIOLOGICAL EXAMINATION OF COLORING SOLUTIONS, FLAVORING EXTRACTS, FRUIT AND NUTS USED IN FROZEN DESSERTS

It has been shown ^{1, 2, 4, 5} recently that the ingredients added to ice cream after the mix has been pasteurized may be a prolific source of bacteria. This is likewise true for all frozen desserts. Some of these ingredients, more especially nuts, may be handled in such a manner as to be potential sources of infection.³ Furthermore, it has been shown ⁷ that pathogenic bacteria, such as *Eberthella typhosa* and *Staphylococcus aureus*, when introduced into coloring materials are capable of surviving in these materials for a period of at least 2 weeks although numbers decreased rapidly during this period.

In view of these facts, it is desirable to check the sanitary quality of the products added to the mix after pasteurization, especially since it has been shown ⁶ that the sanitary quality of the product may be improved without impairing quality.

I. SAMPLES: COLLECTION AND TRANSPORTATION

Screw cap vials or bottles, 1 oz. capacity, may be used for collecting samples of coloring solutions and flavoring extracts. After a vigorous shaking of the container in which the coloring solution or flavoring extract is kept, pour the sample, consisting of about 10 ml., directly into the sample vial in the same manner as used when the ingredient is poured out for use; or the sample may be taken with a sterile glass or metal sampling tube.

For fruit and nut samples, 3 oz. or 4 oz. wide-mouthed, screw-cap jars are desirable. A sterile, long handled spoon of the type used at soda fountains for stirring ice cream sodas is suitable for use in putting the samples into the jars. Each sample shall consist of at least 1 oz. of the ingredient.

While the microorganisms do not multiply as fast in these materials as in milk, it is nevertheless necessary to cool the samples promptly to 32° to 42° F. and hold them at that temperature until analyzed. Record the length of time between the collection of the

samples and the analysis, and the temperature of the samples when examined.

Crushed ice and water or other suitable refrigerant may be used for cooling. Use proper trays, boxes, or other equipment designed for adequate cooling, for transportation of samples at all times. To protect the samples from ice water contamination, place them in water-tight containers. These containers can then be completely covered with cracked ice. For additional details see Part I, A, VI, pp. 11-12.

II. APPARATUS AND MATERIALS

For the description and specifications of the apparatus and materials needed to carry out this method see Part I, B, II, pp. 16-22.

Since fruits and nuts consist of pieces of varying size, it is necessary to use dilution bottles with large openings. Eight oz., wide-mouthed, glass-stoppered or screw-top bottles are convenient. It is also permissible to use half-pint milk bottles, stoppered with a regular paper disc caps. In sterilizing the glass-stoppered bottles, it is advisable to place a piece of paper between the glass stopper and the ground neck of the bottle to prevent vacuum sealing of the bottles when they begin to cool off.

III. PREPARING SAMPLE AND MAKING DILUTIONS

For detailed instructions for making dilutions see Part I, B, IV, 1-6, pp. 25-28. Suggested dilutions for the fruits and nuts are 1:10 and 1:100, for the coloring solutions 1:100 and 1:1000, and for flavoring extracts 1 ml. or 1 gm. direct and 1:10 per ml. or per gm. Since the coloring solutions and flavoring extracts are liquids, they are handled in the same manner as milk samples. In the case of fruits and nuts, it is suggested that 11 gm. samples be weighed directly into dilution bottles and then that 99 ml. sterile water be added. This will give approximately a 1:10 dilution. Allow the mixture to soak about 5 minutes and then shake vigorously 25 times.

IV. AGAR MEDIUM

Use standard nutrient agar. For preparation see Part I, B, III, 1-3, pp. 22-25.

V. PLATING

Use the agar plate method for estimating the bacterial content of these ingredients. The direct microscopic method is not suitable for this purpose. Place 1 ml. of the dilution in each of 2 plates and then use about 10 ml. standard agar to each plate. Mix.

VI. INCUBATION

Follow plating directions given in Part I, B, V, p. 28. Incubate according to the procedure in Part I, B, VI, pp. 29-31.

VII. COUNTING PLATES

All colonies, regardless of size, shall be counted. For the description of the apparatus needed and technic to be followed see Part I, B, VII, 1-6, pp. 31-35.

VIII. REPORTS

Report final results, the average of 2 plates in the form of Standard Plate Count per ml. or per gm. as the case may be. See Part I, B, VIII, pp. 35-37.

IX. TESTS FOR ORGANISMS OF THE COLIFORM GROUP

Organisms of the coliform (*Escherichia-Aerobacter*) group may be found in coloring solutions, and on fruits and nuts. Since these ingredients are handled by humans or are washed in water in which coliform organisms may have grown it may be inferred that the organisms have sanitary significance.^{8, 9} The tests used shall include both the presumptive test and the completed test.

1. MEDIA

The following are recognized as standard media:

- a. Brilliant green lactose peptone bile 2 per cent
- b. Formate ricinoleate broth
- c. Violet red bile agar
- d. Desoxycholate agar

Specific directions for preparing these media are given in Part I, F, V, 1-3, pp. 77-79.

2. PRESUMPTIVE TEST

Consult the directions for making the presumptive test given in Part I, F, VI, 1, pp. 79–80. Inoculate appropriate graduated quantities of liquid materials into fermentation tubes containing brilliant green lactose peptone bile or formate ricinoleate broth. In the case of fruits and nuts, use 10 ml. of the 1:10 dilution for each of the fermentation tubes containing 20 ml. extra strength broth. (Use of 5 tubes for each dilution is recommended.) Incubate these tubes at 37° C. for 48 hours.

The development of gas in 1 of the 5 tubes within this period constitutes a positive presumptive test. If no gas develops in any of the tubes the test is negative and it is concluded that the ingredient is free from coliform organisms in the amounts tested.

3. COMPLETED TEST

Consult the directions for making the completed test given in Part I, F, VI, 2, pp. 80–81. When positive presumptive tests have indicated that gas forming organisms are present it may be desirable to prove that the gas was formed by organisms of the coliform group. The distinguishing characters of coliform organisms are: Aerobic or facultative anaerobic, Gram-negative, non-spore-forming bacteria which ferment lactose with gas formation.

Using a sterile loop, streak an eosin-methylene-blue agar plate with the broth from a positive fermentation tube for a partially confirmed test. After 18–24 hours' incubation at 37° C., pick one or more typical colonies and inoculate each into a tube of lactose broth and onto an agar slope. After 24 hours' incubation the formation of gas in the lactose broth and the determination by microscopic examination from the growth on the agar slope of the presence of Gram-negative, non-spore-forming bacteria constitutes a positive completed test.

X. TESTS FOR HEMOLYTIC STREPTOCOCCI

The hunt for pathogenic microorganisms in foods is always an arduous task. Since these ingredients may be handled by humans, thus presenting opportunities for contamination, different pathogenic and non-pathogenic organisms may be deposited on and in them. The test for hemolytic streptococci is relatively simple and may be.

applied to these ingredients. Follow the directions given in Part I, G, pp. 87-92.

XI. YEAST AND MOLD COUNTS

Yeasts and molds may be present, especially in fruit preparations and on nuts, causing varying degrees of spoilage. These organisms are easily cultured because they grow readily on the ordinary sugar media. They are able to grow and develop colonies in media acidified to a point at which bacteria do not grow. Advantage is taken of this fact in the test. For directions see Part I, J, III, pp. 112-113.

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E. MICROBIOLOGICAL EXAMINATION OF SWEETENING AGENTS USED IN FROZEN DESSERTS

INTRODUCTION

Many kinds of sweetening agents, such as sucrose (beet and cane), lactose, cerelose, invert syrup, corn syrup, maple syrup, liquid sugar, and honey, are used in frozen desserts.*

I. SAMPLES: COLLECTION AND TRANSPORTATION

Remove with a sterile sampling tube or other suitable device $\frac{1}{2}$ lb. samples from each of 5 bags, barrels, or containers of the shipment in question. Sampling from several containers is recommended because of variations that are known to exist in the contamination of containers of sweetening agents representing the same lot. Thoroughly stir the contents of containers of honey or liquid sugar (syrup) before sampling. In no case shall samples for bacteriological analysis be taken only from the uppermost layer.

Record full information regarding the name and type of product; e.g., sucrose (beet or cane), cerelose, etc.; name and address of manufacturer; code number or other lot-identifying mark.

Transport samples to the laboratory as quickly as possible. Do not ice liquid samples but keep them in a cool place and examine them as soon as possible after arrival at the laboratory.

II. APPARATUS AND MATERIALS

Sterilize sampling devices, sample containers, glassware, and other apparatus necessary for analysis according to Part I, A, I, pp. 7-8.

III. PREPARING SAMPLE AND MAKING DILUTIONS

The method of preparing dilutions of the samples shall be by weighing 11 gm. of the sample into a sterile 150 ml. Erlenmeyer flask marked to indicate a volume of 110 ml. Add sterile water to

* The methods given are essentially the same as those accepted tentatively by the Association of Official Agricultural Chemists.^{1, 2, 3}

the 110 ml. mark. A crystalline or amorphous sample can best be weighed aseptically onto a sterile filter paper and then transferred to the dilution flask. A liquid sample can best be weighed directly into the dilution flask.

IV. MEDIA

Express the reaction of any medium in terms of the hydrogen-ion concentration. Either electrometric or colorimetric methods may be used to test and adjust the reaction of media as directed in Part I, B, III, 2, pp. 23-24.

Sterilize and store all media, unless otherwise indicated, according to directions given in Part I, B, III, 3, pp. 24-25.

1. AEROBIC CULTURE MEDIUM

The medium to be used for making plate counts of mesophilic or thermophilic aerobes shall be dextrose tryptone agar containing brom-cresol purple indicator. This medium and the media for the detection of anaerobes are recommended because of the desirability of keeping the media requirements as nearly uniform as possible with those already in use for the examination of sugar. The composition of the medium suggested by the Research Laboratories of the National Canners' Association for the detection of thermophilic bacteria in sugars is given as follows:

Tryptone	10.0	gm.
Dextrose	5.0	"
Agar	15.0	"
Brom-cresol purple	0.04	"
Water	1,000.0	ml.
Final pH 6.7		

In order to obtain uniform results, use *Bacto-dehydrated dextrose-tryptone agar, or its equivalent prepared from products of equivalent quality. Dissolve the agar in a steam chest or autoclave. The remaining ingredients may be added after the agar is dissolved. The brom-cresol purple indicator may be added from a stock solution of such concentration that 1 ml. will contain exactly 0.04 gm. Make up the lost volume with distilled water and test the reaction. The final reaction of the medium shall be approximately pH 6.7. Distribute the medium in the desired containers and sterilize.

* Difco Laboratories, Inc., Detroit, Mich.

2. YEAST AND MOLD CULTURE MEDIUM

Use potato dextrose agar to determine yeast and mold counts. See Part I, J, III, 3-5, pp. 112-113 for details of preparation.

3. ANAEROBIC CULTURE MEDIA

The media recommended for the detection of mesophilic anaerobes shall be uniform with those used¹ for the detection of thermophilic anaerobes. The composition, preparation, and intended uses of these media are given below.

Liver Broth—This medium is intended for the detection of thermophilic anaerobes not producing hydrogen sulfide (*Clostridium thermosaccharolyticum*) and of putrefactive and other mesophilic anaerobes.

In the preparation of this medium, mix chopped beef liver with water in the proportion of 500 gm. to 1,000 ml. Boil this mixture slowly for 1 hour, adjust approximately to pH 7.0, boil for an additional 10 minutes, then press the boiled material through cheese cloth and make up the volume to 1,000 ml. Add 10 gm. tryptone and 1 gm. dipotassium phosphate to the broth. Adjust the reaction to pH 7.0. In tubing, introduce into each tube $\frac{1}{2}$ -1 in. of the previously boiled, ground beef liver.

Immediately before use, unless freshly prepared, the medium shall be heated in streaming steam at least 20 minutes to expel air. Immediately after inoculation, preferably stratify the medium with a 2-2½ in. layer of standard nutrient agar (Part I, Section B, III, 1, pp. 22-23) which has been cooled to 55° C., or stratify with sterile vaseline. If the inoculated medium is to be incubated at 55° C., preheat the tubes in a water bath before placing them in the incubator.

In liver broth at 55° C. thermophilic anaerobes become evident through the splitting of the agar and the production of acid. At times, a cheesy odor is noted. With incubation at 37° C., the presence of putrefactive anaerobes becomes apparent through splitting of the agar and by the presence of a putrid odor.

Sulfite Agar—This medium is intended for the detection of the sulfide-spoilage types of spore-forming organisms (*Clostridium nigrificans*).

This medium is prepared as follows: 1 liter water; 10 gm. tryptone; 1 gm. sodium sulfite (Na_2SO_3); 20 gm. agar. At the time

of tubing, place a clean iron strip or nail in the tube. No adjustment in reaction is necessary.

In sulfite agar the sulfide-spoilage organisms will produce characteristic, blackened, spherical areas, usually without gas formation. Thermophilic anaerobes not of the sulfide-spoilage group will produce gas, coupled with a general blackening of the medium. The darkening of the medium in this case results from the reduction of the sodium sulfite by hydrogen gas. This medium has replaced the sulfite-agar medium in which yeast water was used as the base.²

V. PLATING, INOCULATING AND INCUBATING CULTURES

Examination for Mesophilic Bacteria, Yeasts and Molds—Plate immediately after the dilutions are made. Pour the plates as soon as practicable and certainly within 20 minutes. Prepare two sets of 5 plates each from the diluted sample by transferring 2 ml. of the dilution to each plate. Pour one set of plates with dextrose tryptone agar for the detection and enumeration of the mesophilic bacteria. Incubate these plates at 37° C. for 48 hours. Pour the other set of plates with potato dextrose agar and incubate at 21–25° C. for 5 days for the detection and enumeration of yeasts and molds. Add about 10 ml. liquefied medium at a temperature of 40°–45° C. to the diluted sample in the Petri dish. Observe the usual precautions in plating and pouring plates. Allow the agar to solidify as rapidly as possible after pouring the plates.

Distribute 20 ml. of the diluted sample equally among 6 tubes of liver broth. Likewise distribute 20 ml. of the diluted sample among 6 tubes of melted sulfite agar. Stratify and handle the inoculated tubes as directed above under the heading 3. Anaerobic Culture Media.

VI. COUNTING PLATES

All plates shall be counted according to the methods outlined in Part I, B, VII, pp. 31–35.

Usually a mixture of acid-producing and non-acid-producing bacteria will be encountered in sweetening agents and it is desirable to enumerate the numbers of each type. Acid-producing bacteria are characterized by a yellow halo surrounding the colony in a field of purple. Detection of this type of colony may be facilitated by holding the plate above an illuminated white background.

Growth of anaerobes in tubes of media is detected as described above under 3. Anaerobic Culture Media.

VII. TESTS FOR ORGANISMS OF THE COLIFORM GROUP

To determine the presence or absence of organisms of the coliform group in sugar and sugar products, use the method outlined in Part I, F, pp. 74-85. Since other fermentable carbohydrates will be present, conclusions as to the presence or absence of coliform organisms shall be based only upon the completed test.

VIII. EXAMINATION FOR SPORES OF THERMOPHILIC BACTERIA

At times it is desirable to test sweetening agents to be used in frozen desserts for the presence of spores of thermophilic bacteria. The directions for preparing and plating samples, descriptions of culture media, etc., shall be those recommended by Cameron² for the examination of sugar.

Examination may be made for spores of the following bacteria:

Bacillus stearothermophilus Donk, frequently described as the flat-sour type because it produces acid but not gas in cans of non-acid food.

Clostridium nigrificans Werkman and Weaver, frequently described as the hard-swell or non-hydrogen sulfide type of anaerobe.

The methods to be used for the detection and enumeration of the above organisms are as follows:

Place 20 gm. of the sugar in a sterile 150 ml. Erlenmeyer flask marked to indicate a volume of 100 ml. Add sterile water to the 100 ml. mark. Bring rapidly to boiling, and boil for 5 minutes. Make up for evaporation by adding sterile water.

Detection of Flat-Sour Spores—Pipette 2 ml. of the boiled sugar solution into each of 5 Petri plates. Cover the plate and mix the inoculum with Bacto-dextrose-tryptone agar.* Incubate the plates at 55° C. for 36-48 hours. Humidify the incubator to prevent drying of the agar. The combined count from the 5 plates represents the num-

* This medium may be obtained in dehydrated condition from the Difco Laboratories, Detroit, Mich., or from the various supply houses that carry Difco products in stock. Bacto-dextrose-tryptone agar was developed by the Difco Laboratories in collaboration with the Research Laboratory of the National Canners' Association with this special purpose in mind. It appears peculiarly adapted to growth of flat-sour bacteria. The colonies, both surface and subsurface, exhibit distinctive growth in this medium. Subsurface colonies are larger than in media formerly suggested. Further advantages lie in its standardization and convenience of preparation.

ber of spores in 2 gm. of the original sugar. Multiply this count by 5 in order to express results in terms of Number of Spores per 10 gm. of sugar.

Characteristics of Flat-Sour Colonies—The colony is round, 2–5 mm. in diameter, presents a typical, opaque, central spot and, by reason of acid production in the presence of the indicator, is usually surrounded by a yellow halo in a field of purple. This halo may be insignificant, or missing, with certain low-acid-producing types or when so thickly seeded that the entire plate exhibits a yellow tinge. The typical subsurface colonies are rather compact and may approach pin point size.

At times the nature of the subsurface colonies may be in question. Whether or not they are flat-sour colonies may often be determined by fishing colonies and streaking on dextrose tryptone agar plates. Their surface characteristics may be noted after suitable incubation. If a reasonably pure culture results it is logical to assume that the subsurface colonies have been formed by similar bacterial groups. It is emphasized that when the plate is heavily seeded, the counts may be low and colony structure and size atypical. When plates are so heavily seeded as to make counting impracticable, plate a second sample of the sugar using dilutions of the original solution. For practical purposes it is sufficient to note that the sample is obviously below standard.

Detection of Thermophilic Anaerobes not Producing Hydrogen Sulfide—Divide 20 ml. of the boiled sugar solution among 6 liver-broth tubes and stratify the liquid medium with standard nutrient agar or yeast-water agar. After the agar has solidified, preheat to 55° C. and incubate at that temperature for 72 hours in a humidified incubator.

Under the conditions stated, thermophilic anaerobes become evident through the splitting of the agar and the production of acid. At times a cheesy odor is noted. The method is considered suitable only as a qualitative test since it does not permit expression of results in terms of numbers of spores per unit weight of sugar.

Detection of Thermophilic Anaerobes Producing Hydrogen Sulfide—This group is made up of the so-called sulfide-spoilage organisms. Divide 20 ml. of the boiled sugar solution among 6 tubes containing sulfite agar. Make inoculations in freshly exhausted, deep-agar tubes. Incubate at 55° C. for 72 hours in a humidified incubator.

In sulfite agar the sulfide-spoilage organisms are detected through the formation of characteristic, blackened, spherical areas. Because of the solubility of hydrogen sulfide and its fixation by the iron, no gas is noted. This condition is readily distinguishable from the general blackening produced by certain of the non-hydrogen-sulfide producing thermophilic anaerobes (see above for methods of detection). The discrete blackened areas may be counted to obtain quantitative results.

IX. REPORTS

It is desirable that a uniform system be adopted for reporting the numbers of microorganisms in samples of sweetening agents. The following system of reporting numbers is selected in order to maintain uniformity with the scheme adopted by the Research Laboratory of the National Canners' Association:

Express all results of plate counts in terms of Number of colonies per 10 gm. of the sweetening agent. Record growth in tubes of anaerobic media as + (growth) or — (no growth); e.g., growth in 2 tubes out of 6 shall be recorded as + + — — — —.

Report total counts and flat-sour and sulfide-spoilage results as Number of spores per 10 gm. of sugar. Report thermophilic anaerobes (not producing hydrogen sulfide) as Number of tubes positive and Number of tubes negative, in the following manner: + + + — — —.

Make a uniform record and report of the examination of the samples containing the following information:

Name and type of product [e.g., Sucrose (Beet)]
Manufacturer
Lot or code number of shipment
Date examined
Results

The following standards of the National Canners' Association are appended for the information of public health laboratory workers. They were developed for use in determining suitability of ingredients for use in canned products.

Total Thermophile Spore Count—For the 5 samples examined, there shall be a maximum of not more than 150 spores in any sample and an average of not more than 125 spores per 10 gm. of sugar.

Flat-Sour Spores—For the 5 samples examined, there shall be a maximum of not more than 75 spores in any sample and an average of not more than 50 spores per 10 gm. of sugar.

Thermophilic Anaerobe Spores—These shall be present in not more than 3 (60 per cent) of the 5 samples; and in any sample to the extent of not more than 4 (65 + per cent) tubes.

Sulfide-Spoilage Spores—These shall be present in not more than 2 (40 per cent) of the 5 samples; and in any sample to the extent of not more than 5 spores per 10 gm. This is equivalent to 2 colonies in the 6 inoculated tubes.

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F. THE MICROBIOLOGICAL EXAMINATION OF EGGS AND EGG PRODUCTS USED IN FROZEN DESSERTS

TENTATIVE *

INTRODUCTION

The procedure followed for the microbiological examination of all egg products is essentially the same except with regard to the sampling methods and the preparation of the sample for analysis.¹⁻⁴ The microflora of egg products consists primarily of the common types of microorganisms usually encountered in nature.^{5, 6} Hence the common liquid and plating media are suitable for their cultivation.

Commercial egg products may be classified as follows:

1. Shell eggs
2. Broken-out eggs (liquid eggs):
 - a. Whole or mixed eggs
 - b. Whites (albumin)
 - c. Yolks
3. Dried eggs:
 - a. Whole or mixed eggs
 - b. Whites (albumin)
 - c. Yolks
4. Frozen eggs:
 - a. Whole or mixed eggs
 - b. Whites (albumin)
 - c. Plain yolks
 - d. Sugar yolks
 - e. Salt yolks
 - f. Glycerine yolks

I. SAMPLES: COLLECTION AND TRANSPORTATION

Provide a sufficient number of suitable trays, boxes, cases, or other rigid equipment designed for the adequate and prompt cooling, protection, and transportation of samples of eggs and egg products.

* Lack of time has prevented the circulation of Section F in mimeographed form as a Proposed Method for criticism and comment as has been done in the case of the other methods for examination of frozen desserts and ingredients appearing in this edition; accordingly it is considered as a "Tentative Method." Comments should be sent to the Committee on Standard Methods for Analyzing Frozen Desserts and Ingredients. The methods given are essentially the same as those accepted tentatively by the Association of Official Agricultural Chemists.^{1, 2, 8, 4}

1. SHELL EGGS

Sampling Equipment—

- a. Clean commercial egg case
- b. Record book, pencils, etc.

*Sampling Procedure—*The sample shall consist of a representative number of eggs from the lot to be examined. Select the eggs with aseptic precautions, place them in a clean case and transport them to the laboratory as rapidly as possible. Avoid excessive handling. Keep shell-egg samples cool (40–50° F.) if there is any delay in transportation to the laboratory.

2. BROKEN-OUT EGGS (LIQUID EGGS)

Sampling Equipment—

- a. Alcohol burner
- b. Ethyl alcohol, 95 per cent
- c. Sterile glass or aluminum sampling tubes of uniform bore (0.25 in. x 18 in.), or metal dippers
- d. Sterile 1 pt. or 1 qt. glass jars
- e. Clean towels
- f. Record books, pencils, etc.

*Sampling Procedure—*Take sample from a representative number of cans (the square root of the total number) in the lot to be examined. Note and record all marks of identification. Open containers and remove samples in an aseptic manner. Thoroughly stir the contents of each can of eggs with a sterile sampling tube or dipper. Draw from each can $\frac{3}{4}$ pint of egg material and place in a sterile glass jar. Examine each can of liquid egg material organoleptically after removing the bacteriological sample and record odors as Normal, Putrid, Sour, or Musty.

Refrigerate sample containers with dry ice, or other equally satisfactory refrigerant, if the analysis is to be delayed or if the sampling point is at some distance from the laboratory.

3. DRIED EGGS

Sampling Equipment—

- a. Alcohol burner
- b. Ethyl alcohol, 95 per cent
- c. Grain trier (1 in. x 36 in.)
- d. Sterile 1 qt. glass jars
- e. Absorbent cotton
- f. Clean towels
- g. Record book, pencils, etc.

Sampling Procedure—Take the samples from a representative number of containers (the square root of the total number) of dried eggs in the lot to be examined. Note and record all marks of identification. Open containers in an aseptic manner. Wipe the grain trier with alcohol-soaked cotton and sterilize it in the flame of the alcohol burner unless some other type of flame is more readily available. The sample shall consist of at least 3 cores taken equidistant between the side and the center of the container and separated from each other by one-third of the periphery of the container. Transfer the cores of the dried egg material to the sterile container in an aseptic manner. Between the sampling of each container, wipe the grain trier with a clean towel, sponge it with alcohol and resterilize it.

4. FROZEN EGGS

Sampling Equipment—

- a. Electric or hand drill with auger (1 in. x 16 in.)
- b. Alcohol burner
- c. Ethyl alcohol, 95 per cent
- d. Absorbent cotton
- e. Two tablespoons
- f. Sterile 1 pt. or 1 qt. glass jars
- g. Hammer and steel strip (12 in. x 0.25 in.) or other tool for opening cans
- h. Water pail
- i. Clean towels
- j. Record book, pencils, etc.

Sampling Procedure—Take the samples from a representative number of containers (the square root of the total number) in the lot to be examined. Note and record all marks of identification; *e.g.*, firm name and location, brand, type of product, code or lot number, etc.

Sponge off the auger and the tablespoons with alcohol-soaked cotton and sterilize them by heating them in the flame of the alcohol burner unless some other type of flame is more readily available. After each container has been sampled, wash the auger and the spoons in the pail of water, dry them with a clean towel, and resterilize them. Open the containers in an aseptic manner. Take three drill cores each extending from top to bottom of the can. Take one of these from the center of the can, the second midway between the center and the periphery of the can, and the third from

near the edge of the can. Transfer the drillings from the can to the container with a sterile tablespoon.

Examine the product organoleptically by smelling at the opening of a fourth drill-hole made after the removal of the bacteriological sample. The heat produced by the electric drill intensifies the odor of the egg material, thus facilitating the organoleptic examination. Record odors as Normal, Putrid, Sour, or Musty.

Refrigerate samples with dry ice or other equally satisfactory refrigerant if the analysis is to be delayed or if the sampling point is at some distance from the laboratory. Carry out all sampling procedures under as nearly aseptic conditions as possible.

II. APPARATUS AND MATERIALS

Use standard, calibrated, bacteriological pipettes in the analysis of egg products. Use 150 ml. wide-mouthed, glass- or rubber-stoppered bottles for the preparation of the 1:10 dilution and for higher serial dilutions. Petri dishes shall meet the specifications given in Part I, B, II, 3, p. 19.

Sterilize all sampling devices, sample containers, bacteriological glassware, culture media and other materials necessary for sampling and analysis of egg products, in accordance with Part I, A, I, pp. 7-8; B, II, 4-6, pp. 19-20; B, III, 3, p. 24.

III. PREPARING SAMPLE AND MAKING DILUTIONS

1. SHELL EGGS

Wash the eggs with a stiff brush, using soap and warm water. The washed eggs may be drained on a wire test tube rack. Immerse the washed eggs in 70 per cent ethyl alcohol for 10 minutes. Remove each egg from the alcohol, drain it well, and flame it before opening. Grasp the egg by the blunt or air-cell end and puncture a 0.5 in. hole in the acute end with a sterile scalpel or with sterile forceps. Flame the shell opening before removing the contents of the egg. Invert the opened egg over a crucible triangle placed on a tripod and force the contents into a wide-mouthed, glass- or rubber-stoppered bottle by heating the air-cell end of the egg with a Bunsen flame. Thoroughly mix the contents into an egg batter with a sterile, electrical stirrer.

Prepare a 1:10 dilution by aseptically weighing 5.0 gm. of egg material into a sterile, wide-mouthed, glass- or rubber-stoppered

bottle containing 45 ml. sterile distilled water and 1 tablespoonful of glass shot. Thoroughly agitate the 1:10 dilution to insure complete solution or distribution of the egg material in the diluent by shaking the container rapidly 100 times, each shake being an up-and-down excursion of about 1 foot, the time interval not exceeding 45 seconds. Use sterile distilled water in the preparation of serial dilutions. Use the 1:10 dilution as a basis for the preparation of all higher serial dilutions necessary in the analysis of any given sample.

2. BROKEN-OUT EGGS (LIQUID EGGS)

Thoroughly mix the broken-out eggs into an egg batter with a sterile spoon or an electric stirrer. Prepare a 1:10 dilution by weighing 11 gm. of egg material aseptically into a wide-mouthed, glass- or rubber-stoppered bottle containing 99 gm. sterile water and 1 tablespoonful of sterile glass shot. To insure complete solution and distribution of the egg material in the diluent, thoroughly agitate this 1:10 dilution by shaking the container rapidly 100 times, each shake being an up-and-down excursion of about 1 foot, the time interval not exceeding 45 seconds. Use the 1:10 dilution as a basis for the preparation of other necessary serial dilutions, from 1:10 to 1:1,000,000,000. Use sterile distilled water as the diluent in the preparation of all dilutions.

3. DRIED EGGS

Egg Whites (Albumin)—Thoroughly mix the sample with a sterile spoon or spatula. Weigh 11 gm. of dried egg white into a sterile mortar and grind with a sterile pestle until a fine powder is obtained. Intermittently add the contents of a 99 ml. sterile, distilled water dilution blank to the ground egg white in the mortar, grinding continually until the entire contents of the dilution blank have been used. The resulting suspension is a 1:10 dilution of dried egg white which shall be used as the basis for the preparation of other necessary serial dilutions, following the directions given above for liquid eggs.

Dried Whole Egg and Dried Egg Yolk—Thoroughly mix the sample with a sterile spoon or spatula. Prepare a 1:10 dilution of these products in the manner directed for frozen eggs, unless the material is quite insoluble; in that case prepare the 1:10 dilution as

directed for dried egg white. Use this 1:10 dilution as the basis for the preparation of other necessary serial dilutions, following the directions given above for liquid eggs.

4. FROZEN EGGS

Thaw frozen egg material as rapidly as possible to prevent increases in the numbers of microorganisms present, yet at a temperature sufficiently low to prevent their destruction. A temperature of 37° C. is recommended. Frequent rotary shaking of the sample container will aid in the thawing of the frozen material. Thawing temperatures may be maintained by the use of a water bath or a bacteriological incubator. After it has become completely thawed, thoroughly mix each sample with a sterile spoon or an electric stirrer. Prepare all serial dilutions (1:10 to 1:1,000,000,000) necessary for the examination of any particular sample, following the directions given above for liquid eggs.

IV. MEDIA

State the reaction of culture media in terms of hydrogen-ion concentration as expressed in pH values. Tests to control the adjustment to the required hydrogen-ion concentration may be made by either electrometric or colorimetric methods as directed in Part I, B, III, 2, p. 23.

1. STANDARD NUTRIENT AGAR

Use either the old or the new standard nutrient agar. Directions for making the old standard agar will be found in Standard Methods for Milk Analysis, 6th Ed., Part 1, C, IV, pp. 16-19, 1934, or in Standard Methods of Water Analysis, 8th Ed., Part VII, III, G, p. 201, 1936. This agar is composed of 3 gm. beef extract, 5 gm. Bacto-peptone and 15 gm. undried shredded agar added to 1,000 ml. distilled water. Reaction is to be adjusted so that the pH reading after sterilization will be between 6.4 and 7.0.

Directions for making the new standard agar are given in Part I, B, III, pp. 22-25. The new agar differs from the old in that it contains Bacto-tryptone in place of Bacto-peptone. Glucose is added to the amount of 0.1 per cent. No skim milk need be added

where this agar is used for analyzing egg products. Preferred reaction ph 7.0.

2. LACTOSE BROTH

This is prepared from standard nutrient broth by adding 5 gm. or 10 gm. as preferred of lactose per liter. The peptone used may be either Bacto-peptone or Bacto-tryptone. (Part I, B, II, 13, p. 22.)

3. ENDO AGAR

FORMULA I

1. *Preparation of Stock Agar.* Add 5 gm. of beef extract, 10 gm. of Bacto-peptone and 30 gm. of agar to 1,000 ml. of distilled water. The undried market type of agar as stored in the ordinary laboratory may be used if desired.

Boil until the agar is dissolved and make up lost weight due to evaporation, with distilled water.

Adjust the reaction so that the pH reading after sterilization will be 7.4.

Clarify if desired.

Add 10 gm. of lactose and dissolve.

Place in small flasks or bottles 100 ml. to each and sterilize in the autoclave at 15 lbs. (121° C.) for 15 minutes after the pressure has reached 15 lbs.

2. *Preparation of Plates.* Prepare a 3 per cent solution of certified basic fuchsin in 95 per cent ethyl alcohol.

Allow to stand 24 hours and filter.

Melt lactose agar as prepared above and to each 100 ml. add 1 ml. of the 3 per cent basic fuchsin solution and 0.125 gm. of anhydrous sodium sulfite dissolved in 5 ml. of distilled water. The sulfite solution must be freshly prepared.

Mix thoroughly, pour plates with usual precautions against contamination and allow to harden.

The medium should be light pink when hot and almost colorless when cool. As batches of fuchsin differ somewhat in dye content, it is possible that the medium made up according to this formula may be too highly colored before incubation or may not give the proper reaction when seeded with coliform bacteria. In such a case, the strength of the basic fuchsin solution may be varied.

FORMULA II

Dipotassium phosphate K_2HPO_4	3.5	gm.
Bacto-peptone	10.0	gm.
Agar (washed and dried)	15.0	gm.
Water (distilled)	1	liter
Lactose (C.P.)	10.0	gm.

To each 100 ml. of the above add:

Sodium sulfite (anhydrous)	0.25	gm.
Basic fuchsin (pararosanilin), filtered 10 per cent alcoholic solution *	0.5	ml.

* Some basic fuchsin is not soluble enough to make a 10 per cent alcoholic solution.

4. EOSIN-METHYLENE-BLUE AGAR

Add 10 gm. of Bacto-peptone, 2 gm. of dipotassium phosphate (K_2HPO_4) and 15 gm. of undried agar to 1,000 ml. of distilled water.

Boil until all ingredients are dissolved and make up loss due to evaporation with distilled water.

Adjustment of reaction is not necessary.

Place measured quantities (100 or 200 ml.) in flasks or bottles and sterilize in the autoclave at 15 lbs. (121° C.) for 15 minutes after the pressure has reached 15 lbs.

To prepare plates, melt stock agar as described above and to each 100 ml. add 5 ml. of sterile 20 per cent aqueous lactose solution, 2 ml. of 2 per cent aqueous solution of eosin, yellowish, and 2 ml. of 0.5 per cent aqueous solution of methylene blue.

Mix thoroughly, pour into Petri dishes and allow to harden.

It is permissible to add all of the ingredients to the stock agar at the time of preparation, place in tubes or flasks, and sterilize. Plates may be prepared from this stock. Discoloration of the medium occurs during sterilization. The color returns after cooling.

5. INDOL TEST

(a) *Tryptone Broth.*

1. To 1,000 ml. of distilled water add 10 gm. of Bacto-tryptone (formerly tryptophane broth) and heat with stirring to obtain complete solution.

2. Distribute in 5 ml. portions into test tubes and sterilize in the autoclave at 15 lbs. (121° C.) for 15 minutes after the pressure has reached 15 lbs.

(b) *Reagent*. The test reagent is made up by dissolving 5 gm. of C. P. para dimethyl-amino benzaldehyde in 75 ml. of amyl alcohol and adding 25 ml. of concentrated hydrochloric acid. Some brands of C. P. amyl alcohol are not satisfactory. Laboratory reagent amyl alcohol is recommended. This reagent should have a yellow or light brown color when made up.

(c) *Procedure*. Inoculate 5 ml. portions of medium. Incubate at 37° C. for 24 hours. Add 0.2 to 0.3 ml. of the amyl alcohol indol reagent and shake. Let tube stand for about a minute and observe the result. Dark red color in the amyl alcohol surface layer is a positive indol test, original color of the amyl alcohol reagent is a negative test.

6. METHYL RED TEST

(a) *Peptone Medium*.

1. To 800 ml. of distilled water add 5 gm. of Bacto-proteose peptone or Witte's peptone (other peptones should not be substituted), 5 gm. C. P. dextrose, and 5 gm. dipotassium hydrogen phosphate (K_2HPO_4). A dilute solution of the K_2HPO_4 should give a distinct pink with phenolphthalein.

2. Heat over steam, with occasional stirring, for 20 minutes.

3. Filter through folded filter paper, cool at 20° C., and dilute to 1,000 ml. with distilled water.

4. Distribute 10 ml. portions in sterilized test tubes.

5. Sterilize by the intermittent method for 20 minutes on three successive days.

(b) *Indicator Solution*. Dissolve 0.1 gm. methyl red in 300 ml. alcohol and dilute to 500 ml. with distilled water.

(c) *Procedure*. Inoculate 10 ml. portions of medium. Incubate at 37° C. for 3 days or preferably 4 days. To 5 ml. of the culture, add 5 drops of methyl red indicator solution.

(d) Record distinct red color as methyl red +, distinct yellow color as methyl red —, and intermediate as ?.

7. VOGES-PROSKAUER TEST

(a) *Procedure.* This test may be made on a 5 ml. portion of the medium inoculated for the methyl red test or on a separately inoculated tube of the same medium. In any case the test should be made after 24 to 48 hours of incubation at 37° C. Longer incubation periods tend to reduce the number of positive Voges-Proskauer results due to the destruction of acetyl methyl carbinol. If the test is to be made on the culture inoculated for the methyl red test, 5 ml. of this medium should be withdrawn aseptically after 24 or 48 hours at 37° C. for the Voges-Proskauer test and the tube returned to the incubator to complete the proper incubation time for the methyl red test. Add 5 ml. of a 10 per cent solution of potassium hydroxide solution to the 5 ml. portion or to a separate 5 ml. culture.

(b) *Technic for Hastening the Voges-Proskauer Reaction.* To hasten the production of color the tubes treated with potassium hydroxide are placed in a 37° or 45° C. incubator for six hours. Observations should be made periodically during this period. A positive test is indicated by an eosin pink color. If it is desired the Werkman technic of hastening the color reaction may be used. Two drops of a 2 per cent aqueous solution of ferric chloride are added to 5 ml. of culture, followed by 5 ml. of 10 per cent potassium hydroxide solution. The tube is shaken and put into the incubator for one hour. Readings are then made.

8. SODIUM CITRATE TEST

(a) *Medium.* Dissolve 1.5 gm. sodium ammonium phosphate (microcosmic salt), 1 gm. potassium dihydrogen phosphate, 0.2 gm. magnesium sulfate and 2.5 to 3.0 gm. sodium citrate (crystals) in 1 liter of distilled water. Distribute into test tubes in 5 ml. amounts and sterilize in the autoclave at 15 lbs. (121° C.) for 15 minutes after the pressure has reached 15 lbs.

(b) *Procedure.* Inoculate pure cultures into this medium with a needle or a standard loop. A pipette should never be used on account of the danger of invalidating the result due to the introduction of nutrient material with the transfer. Incubate at 37° C. for 72 to 96 hours, and record growth as + or —.

9. POTATO DEXTROSE AGAR

Prepare as directed in Part I, J, III, 3, p. 112.

10. VEAL INFUSION AGAR

Prepare as directed, Part I, G, I, 2, B, p. 88.

11. HOLMAN'S ALKALINE COOKED MEAT MEDIUM

The composition of this medium is as follows:

Ground fresh lean beef.....	500 gm.
Distilled water	1,000 ml.
Peptone (Bacto)	5 gm.
Sodium chloride, C.P.....	5 gm.

Add the beef to the distilled water and infuse over night in the refrigerator. Skim off the fat. Strain the infusion through several layers of cheese cloth and press out the broth, retaining the meat press cake. Make up the broth to the original volume. Add the peptone. Heat in an Arnold sterilizer for 10 minutes. Filter. Add the sodium chloride. Add normal sodium hydroxide until the medium is alkaline to phenolphthalein. Heat in the Arnold sterilizer for 15 minutes to clear; then filter. Distribute the pressed-out beef remaining from the infusion, approximately 2 gm. to a tube, into test tubes (150 × 20 mm.). Add 10 ml. of the clear alkaline broth to each tube. Sterilize in the autoclave at 15 lb. pressure (121° C.) for 20 minutes. Final reaction pH 7.2–7.4. Prior to use, boil the tubed medium for at least 15 minutes, to expel absorbed oxygen; then cool promptly in a water bath. Store all media in a refrigerator at 40–45° F.

V. PLATING

Examine all egg products by the same analytical procedure. Add 1 ml. portions of all dilutions from 1:10 to 1:1,000,000 to duplicate sets of Petri plates. Pour nutrient agar previously cooled to 41–42° C. Pour all plates and inoculate all media within 20 minutes after the preparation of the first dilution to prevent growth or death of microorganisms in the dilution bottles or tubes.

For comparative purposes two media are suggested: Standard nutrient agar as used for water analysis and standard nutrient agar as used for milk analysis. It is suggested that whenever possible

duplicate platings be made on each medium, that plates be incubated at 20°, at 32° and at 37° C. and, since this method is still tentative, that the results be reported to the Committee on Standard Methods for Analyzing Frozen Desserts and Ingredients.

VI. INCUBATION

One set of plates shall be incubated at 37° C. for 2 days, and other sets at temperatures of 20° C. and/or 32° C. for 3 days. Where incubators cannot be regulated to maintain these temperatures, incubate at room temperature. In general, higher counts are obtained on plates incubated at 20° and 32° than on plates incubated at 37° C.

VII. COUNTING PLATES

In counting plates follow the procedure outlined in Part I, B, VII, 1-6, pp. 31-35, bearing in mind that the plan of counting only those plates containing between 30 and 300 colonies cannot always be followed because of the small number of colonies found in shell eggs and other egg products of good quality. With samples containing insoluble material which does not form a uniform suspension with the diluent, extreme caution should be exercised in counting the plates in order to differentiate between bacterial colonies and masses of insoluble particles from the sample. When in doubt whether or not particles are bacterial colonies, examine them with higher magnification.

VIII. REPORTS

Report results as the Standard Plate Count per gram of the particular product under examination. For additional details see Part I, B, VIII, pp. 35-37.

IX. MICROSCOPIC COUNTS

Place 0.01 cc. of the 1:10 or 1:100 dilutions of egg material on a clean, dry, microscope slide and spread over an area of 1 sq. cm. Dry the smear on a level surface at 35°-40° C. Immerse slides in xylene for 1 minute and dry in air. Immerse slide in 90 per cent ethyl alcohol 1 minute and dry in air. Stain smears for 15 seconds with North's anilin oil methylene blue.* (Part II,

* The anilin oil methylene blue stain was first recommended by W. R. North, Jr., of the Division of Bacteriology, U. S. Food and Drug Administration, Washington, D. C. This stain is very lightly absorbed by the egg solids in a smear, while the microorganisms in the preparation take a very deep blue color, thus facilitating their recognition.

2, C, IX, p. 175.) Wash stained preparations by repeated immersion in a beaker of water. Dry thoroughly before examination. Subsequent procedure and precautions should be observed as directed in Part I, C, II-V, pp. 44-55.

Multiply total count of individual bacteria by 10 or 100, when the original preparation is made from a 1:10 or 1:100 dilution. Express results as numbers of bacteria per gram of egg material.

X. TESTS FOR ORGANISMS OF THE COLIFORM GROUP

The coliform group is considered as including all aerobic or facultative anaerobic, Gram-negative, non-spore-forming bacteria which ferment lactose with gas formation.

1. PRESUMPTIVE TEST

The presumptive test for detecting the presence of the coliform group described in Part I, F, VI, 1, pp. 79-80, and the presumptive test in *Standard Methods for the Examination of Water and Sewage*, 8th Ed., Part VII, Section IX, B, pp. 211-212 may be used as alternate procedures until accumulated data show which is superior for use with egg products. Because egg products, like milk, contain more organic materials than water and so support a more abundant and varied bacterial flora, it seems probable that a broth containing some inhibiting material such as bile or ricinoleate will yield more positive presumptive tests than the plain lactose broth used for testing water. Whenever possible, use more than one procedure and report the comparative results to the Committee on Standard Methods for Analyzing Frozen Desserts and Ingredients.

Inoculate 1.0 ml. portions from suitable serial dilutions (1:10 to 1:100,000,000) of egg material into fermentation tubes of lactose broth. Incubate the inoculated lactose broth tubes at 37° C. for 24 hours. Streak all lactose broth cultures showing gas production onto Levine's eosin-methylene-blue agar or on Endo agar plates. Incubate plates of differential media at 37° C. for 24 hours and examine for the presence of microorganisms of the coliform group. Record the reciprocal of the highest dilution showing positive confirmation on differential media as the number of organisms of the coliform group per gram.

2. COMPLETED TEST

Inoculate nutrient agar slants from typical, well isolated colonies of all the coliform types of bacteria appearing on the differential agar plates. Incubate the inoculated agar slants at 37° C. for 24 hours. Purify slant cultures for further study. Determine the biochemical reactions of the purified cultures according to the methods that are described in Part II, 2, F, IV, 5-8, pp. 198-200. These biochemical tests include Kovac's test for indol production, the methyl red (M.R.) reaction, the Voges-Proskauer (V.P.) reaction and Koser's sodium citrate test for the utilization of sodium citrate as the sole source of carbon.

XI. TESTS FOR HEMOLYTIC STAPHYLOCOCCI AND STREPTOCOCCI⁷

Inoculate Petri plates with 1 ml. portions of all dilutions of egg material from 1:10 to 1:1,000,000. Immediately pour the plates with veal infusion agar containing 5 per cent of defibrinated horse-, sheep-, or rabbit-blood (0.5 ml. blood per 10 ml. medium). Cool the veal agar to 40° C. and add the blood just prior to pouring of the plates. Incubate the inoculated veal agar plates at 37° C. for 24 hours. Confirm presence of coccus types of microorganisms by microscopic examination of smears taken from representative hemolytic colonies and stained by Gram method. Express total counts of hemolytic microorganisms as number of hemolytic staphylococci per gram, or number of hemolytic streptococci per gram of the material under examination.

XII. TESTS FOR PUTREFACTIVE ANAEROBES⁸

Immediately before use, heat tubes of Holman's alkaline meat medium in streaming steam for at least 20 minutes ("exhaust") and then quickly cool them in a water bath. Inoculate a tube of meat medium with a 1 ml. portion of each dilution of egg material (1:10 to 1:100,000). Incubate the inoculated tubes at 37° C. for 3-4 days. Evolution of gas and digestion of the meat indicates the presence of putrefactive anaerobes. Confirm cultures by microscopic examination of smears stained with Gram stain. Record the reciprocal of the highest dilution showing positive anaerobic growth as the number of putrefactive anaerobes per gram of egg material.

XIII. TEST FOR FUNGI 9, 10, 11

Inoculate Petri plates with a 1 ml. portion of each dilution of egg material (1:10 to 1:100,000,000). Immediately pour inoculated plates with potato dextrose agar previously cooled to 40–45° C. Express total counts of fungi as number of fungi per gram of the particular egg material.

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3. SEDIMENT TESTING OF FROZEN DESSERTS AND INGREDIENTS USED IN FROZEN DESSERTS

INTRODUCTION

The sediment test has been applied and considered in connection with milk and cream for many years and the sediment in milk has generally been associated with the dirt commonly found on the farm.¹ As the name implies, sediment refers to the visible particles which settle out and thereby impair the attractiveness of the finished product. Consequently, any particles of dirt in milk have generally been interpreted to indicate that the product is not clean. However, any visible particles that are foreign to the product can properly be considered to be a contamination and may be classified as sediment. This latter viewpoint should be maintained because technically "the matter which settles to the bottom from a liquid," as Webster defines sediment, cannot be applied to frozen desserts as their semiplastic character prevents any settling of particles. With these thoughts in mind, the following definition is proposed:

Definition of Sediment—Sediment in frozen desserts shall be understood to be all foreign matter which is deposited on a sediment test disc, not including any partially or wholly insoluble ingredient normally present in frozen desserts as a result of correct processing or manufacture.

A. SAMPLES AND THEIR PREPARATION

In developing methods for the determination of sediment in frozen desserts, it is advisable to follow, as closely as reliability will permit, methods that are already established and understood. The analysis of frozen desserts necessitates the examination of a variety of milk products which have undergone varying degrees of processing.

The wide variation in the nature and properties of the ingredients used in the manufacture of frozen desserts makes it difficult to designate a single unit of measurement which can be regarded as standard. The same situation prevails with respect to the prepa-

ration of samples for application of the sediment test. Accordingly, the ingredients seem to classify themselves readily into (1) those which closely resemble milk, (2) those which do not resemble milk but which can be readily prepared for sediment testing, and (3) those which require special methods of preparation.

I. SAMPLING MILK PRODUCTS

a. *Milk, Skim Milk, and Cream*—Use pint samples for examination (Part I, E, I, p. 68).

b. *Evaporated Milk*—Use $\frac{1}{2}$ pint samples, adding 1 pint of filtered water to such samples. Stir thoroughly to insure complete mixing.

c. *Sweetened Condensed and Skimmed Condensed Milks*—Use $\frac{1}{2}$ pint samples, adding 1 pint of filtered water. Stir thoroughly to insure complete mixing.

d. *Dry Whole Milk (Exclusive of Roller-process Powder)**—Use samples consisting of a dilution of 50 gm. in 1 pint of filtered water. Completely dissolve the powder by mixing thoroughly.

e. *Dry Skim Milk (Exclusive of Roller-process Powder)**—Use samples consisting of a dilution of 50 gm. in 1 pint of filtered water. Completely dissolve the powder by mixing thoroughly.

f. *Butter*—Use one hundred gram (1 oz.) samples. To such a sample add $\frac{1}{2}$ pint of hot filtered water (70°C.) and heat the mixture on the water bath to a temperature of 65°C. , stirring thoroughly to insure complete melting and mixing.

If the above procedure fails to prepare the butter sample for the sediment test properly, substitute for the $\frac{1}{2}$ pint of hot filtered water, 200 ml. hot, filtered, hydrochloric acid solution prepared by making up 3.2 ml. concentrated C. P. grade hydrochloric acid to 1 liter with filtered, distilled water.

g. *Frozen Dessert Mix*—Use $\frac{1}{2}$ pint samples. To such sample add 1 pint of filtered water, stirring thoroughly to insure complete mixing.

h. *Frozen Dessert*—Use 1 pint samples. To such sample add 1 pint of hot filtered water (70°C.) and heat mixture on water bath to temperature of 65°C. , stirring thoroughly to insure complete melting and mixing.

* Methods for sediment testing of roller process powders are now under consideration by the Committee on Standard Methods for Analyzing Frozen Desserts and Ingredients.

II. SAMPLING INGREDIENTS OF FROZEN DESSERTS

a. *Gelatin, Sodium Alginate, and Other Stabilizers*—Use 5 gm. samples. Soak such samples in 1 pint of cold filtered water for $\frac{1}{2}$ hour, although it may be necessary to soak sodium alginate stabilizers 24 hours or longer. Heat mixture on water bath to a temperature of 65°C ., stirring thoroughly to insure complete mixing in preparing for the sediment test.

b. *Egg Yolks (Fresh or Frozen)*—Use 25 gm. samples. Transfer sample to a 1 liter, glass-stoppered bottle containing 475 ml. (1 pint) previously filtered sodium chloride solution. (If the yolks are sugared yolks, use 9 per cent sodium chloride solution.) Add approximately 25 gm. of glass beads and shake the bottle thoroughly for 1 minute to insure complete mixing.

c. *Whole Eggs (Fresh or Frozen)*—Use 25 gm. samples. Mix sample thoroughly and bring to room temperature. Transfer sample to a 1 liter, glass-stoppered bottle containing 475 ml. (1 pint) 1 per cent aqueous solution of pepsin adjusted to pH 3.0, and incubate at 37°C . for 1 hour, shaking the contents at 15 minute intervals. Add approximately 25 gm. of glass beads and shake the bottle thoroughly for 1 minute to insure complete mixing.

d. *Sugar, Invert Sugar, Vanilla, Sugar Syrups and Salt*—Use 50 gm. samples. To such a sample add 1 pint of filtered water and stir thoroughly to insure complete mixing.

III. SAMPLING FLAVORING MATERIALS

a. *Fruits and Nutmeats*—Use 15 gm. samples which have been previously ground in a mortar with pestle. Weigh the sample in a 50 ml. conical sediment tube. Add 15 ml. distilled, filtered water and shake to make a well mixed emulsion. Add 15 ml. chloroform and shake vigorously for $2\frac{1}{2}$ minutes. Place in centrifuge and whirl for 15 minutes or longer to allow for separation of sand and other dirt particles from the emulsified mixture. The fruit fibers may be floated off and the heavy particles such as sand, dirt, glass, and metal in the chloroform layer may then be mixed with $\frac{1}{2}$ pint hot, filtered water and stirred thoroughly to insure complete mixing.

b. *Puree and Ground Fruits*—Use 15 gm. samples which have been previously ground in a mortar with pestle. Weigh the sample in a 50 ml. conical sediment tube. Add 15 ml. filtered, saturated sodium chloride solution and shake to make a well mixed emulsion

Add 15 ml. chloroform and shake vigorously for $2\frac{1}{2}$ minutes. Place in a centrifuge and whirl for 15 minutes or longer to allow for separation of sand and other dirt particles from the emulsified mixture. The fruit fibers may be floated off and the heavy particles such as sand, dirt, glass, and metal in the chloroform layer may then be mixed with $\frac{1}{2}$ pint of hot, filtered water and stirred thoroughly to insure complete mixing.

B. METHOD OF MAKING SEDIMENT TESTS

I. PREPARATION FOR MAKING SEDIMENT DISCS

Strain the samples of the different materials or ingredients prepared in accordance with the methods outlined above through firm cotton discs placed on openings with an effective filtering area 1 in. in diameter. The straining of samples through cotton discs will be facilitated and the process hastened if, prior to their testing, provision is made for warming the samples prepared as outlined above.

Pressure type sediment testers are more convenient and effective than the gravity type. The hand-operated, vacuum type of sediment tester is not recommended because of the possibility that sediment may be passed around the rim of the plunger or piston of the tester to which the sediment disc is affixed, particularly if the plunger is not withdrawn from the cylinder in the proper manner. Then, too, while this type of tester is probably not as cumbersome to operate as is the pressure type, its efficiency is subject to the criticism that invariably a pool of milk remains on top of the sediment disc which, when emptied off, carries with it sediment still floating or suspended in that portion of the sample.

It is essential that the sediment tester and disc be rinsed with about $\frac{1}{2}$ pint hot, filtered water (70° C.) after the sample has been tested. This will insure removing traces of the sample, and wash down sediment which may adhere to the inside walls of the sediment tester. It is essential in testing egg products to use sufficient water for washing the sediment tester and the filter disc until they become free from foam. Use only filtered distilled or filtered tap water for reconstitution, dilution or rinsing purposes.

II. PREPARATION OF STANDARD SEDIMENT DISC

In the interest of uniformity, discs should be rated by comparing them with standard discs or a photographed standard. An excellent set of standard discs has been prepared and photographed by the Committee on Standard Methods for Analyzing Frozen Desserts and Ingredients in collaboration with the Connecticut State Department

of Health.^{2, 3, 4} This is a modification of the milk sediment test standard to permit its use in evaluating the sediment testing of frozen desserts and their ingredients. See Part I, E, IV, p. 71.

III. METHOD OF RECORDING RESULTS

Photographed copies of standard discs for grading the sediment obtained in applying the sediment test to frozen desserts and ingredients, as outlined above, may be secured through the office of the American Public Health Association, 1790 Broadway, New York, N. Y., at a cost of \$1.00 each. It is urged that all laboratories use such photographed standards rather than attempt to prepare their own standard discs. Photostatic reproductions of the photographed standards are not satisfactory for grading discs and cannot be recommended.

The standards given are based on 1 pint samples of milk to which weighed amounts of sediment have been added. Numerical ratings only are given for the convenience of those who may wish to use them. No attempt should be made to grade as sediment any hair, insect or insect parts, piece of hay or straw, or any large particle of dirt. These should be reported separately as additional notations.

IV. METHOD OF USING DISCS

After recording the sediment score or cleanliness rating (the latter on the basis of arbitrary definition) for each disc, the discs may be prepared for return by mail to the processor of frozen desserts as a matter of record or for filing. This may be done by drying the disc and placing it in a transparent cellophane envelope slightly larger than the disc. It is sometimes advisable to fasten the disc to a small mounting card by means of an office staple which will serve to keep the disc and envelope in good condition. Before placing the disc on the mounting card or in a cellophane envelope, it should first be dried, precautions being taken to protect it from contamination by dust. This can readily be done by placing the disc on a paper towel and covering with another paper towel to prevent contamination.

V. REPORTS

Experience has shown that one of the most effective ways of reporting results to a processor of frozen desserts is to send him

the disc with a statement as to the way in which it was obtained. No reference should be made to the influence of dirt upon the bacterial count as this has not been substantiated by investigation. Dirt of any sort is sufficiently objectionable in frozen desserts or any other food to be condemned solely on the ground that it is dirt.

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PART III. BIOASSAY OF VITAMIN D MILK

FOOD AND NUTRITION SECTION

American Public Health Association

The method herein described incorporates the principles of the method outlined in the *U. S. Pharmacopoeia, 1937 Supplement*, page 91, for the vitamin D assay of cod liver oil. Terms herein used are defined in the U. S. P. Supplement. Certain modifications of the U. S. P. method suggested by the Association of Official Agricultural Chemists¹ for the assay of vitamin D milks are optional with the assayer. The method is essentially the same as that adopted by the Association of Official Agricultural Chemists.

A. COLLECTION AND PRESERVATION OF THE SAMPLE

The sample of milk in the original bottle shall be shipped to the assayer immediately after collection. Shipments shall be made in iced containers. Experience has demonstrated that dry ice is not suitable for refrigeration of milk during shipment unless care is exercised in packing. Even during the summer season the super cooling effect of dry ice in many instances causes the milk to freeze, resulting in the bottles cracking and a total loss of the sample. Shipping containers with icing pans (using ordinary ice) are preferable.

After receipt and acceptance by the assayer, the milk shall be immediately placed on assay or as soon thereafter as possible. If necessary to delay the assay, the milk shall be preserved in its original homogeneous state. This may be accomplished by the addition of two drops of 10 per cent formaldehyde per quart of milk and ordinary refrigeration (40°–50° F.) for a period of not more than 30 days. Once a sample of milk becomes soured, curdled, or the fat definitely separated out, it is unsuitable for assay purposes. Obviously, collection and preservation of evaporated or dried milk samples present no special problems; however, once they have been submitted to the assayer and opened, precautions with respect to preservation outlined above must be followed.

B. PRELIMINARY PERIOD

Throughout the preliminary period, each rat shall be raised under the immediate supervision of or according to directions specified by the assayer. Throughout the preliminary period, the rats shall be provided with adequate amounts of a ration which will allow for normal development in all respects. The supply of vitamin D in the diets listed below is sufficient for maintenance needs and is limited to such a degree that rats weighing between 44 and 60 gm. at an age of 21–28 days, when placed on a suitable rachitogenic diet for 3 weeks, should manifest evidence of severe rickets.

The following ration is an example of one suitably low in antirachitic potency, for this purpose:

Yellow maize	71.0
Linseed oil meal	16.0
Crude casein	5.0
Alfalfa meal	2.0
Butter fat	5.0
Bone ash	0.5
Sodium chloride	0.5
Fed with fresh whole milk and water <i>ad libitum</i>	

If it is not convenient to use fresh milk, the diet may be modified as follows:

Yellow maize	57.0
Whole milk powder	25.0
Linseed oil meal	12.0
Crude casein	3.7
Alfalfa leaf meal	1.5
Iodized sodium chloride	0.4
Calcium carbonate	0.4
With water <i>ad libitum</i>	

All the solid constituents of the ration must be finely ground so as to prevent selection by the animals. Care must be exercised that milk and butter fat are used to which an antirachitic substance has not been added.

C. DEPLETION PERIOD

A rat shall be suitable for the depletion period when the age of the rat does not exceed 28 days, and if the body weight of the rat shall exceed 44 gm. and shall not exceed 60 gm., and if the animal manifests no evidence of injury, disease, or anatomical abnormality which might hinder growth and development. Throughout the depletion, each rat shall be provided with the rachitogenic diet and distilled water *ad libitum*, and during this period no other dietary supplement shall be available to the animal. When the rats have reached the weight and age range above indicated, they are made rachitic by transference to and maintenance on a very finely ground rickets producing diet. Formulae for suitable rachitogenic diets are given below.

McCollum Diet No. 3143²

No. 1	Whole yellow maize ground.....	33.0
	Whole wheat ground.....	33.0
	Ground gluten	15.0
	Gelatin	15.0
	Calcium carbonate (CaCO_3).....	3.0
	Sodium chloride (NaCl).....	1.0

Steenbock Diet No. 2965³

No. 2	Whole yellow maize ground.....	76.0
	Ground gluten	20.0
	Calcium carbonate (CaCO_3).....	3.0
	Sodium chloride (NaCl).....	1.0

It is advisable that each new lot of ration be used in a preliminary way to determine whether it produces satisfactory rickets.

Throughout the depletion and subsequent assay period, the rats shall be maintained on wire screens in a room away from direct or reflected sunlight of sufficient intensity to influence rickets in the rat. A range of temperature between 72° and 80° F. is satisfactory for the care of the animals during the depletion and subsequent test period.

D. ASSEMBLING RATS INTO GROUPS FOR ASSAY PERIOD

For each milk assay there shall be one or more assay groups. In the assay of one vitamin D milk, there shall be provided at least one reference group, but a single reference group may be used for the concurrent assay of more than one vitamin D milk. On any one day during the interval of assembling rats into groups, the total number of rats that shall have been assigned to make up any one group shall not exceed by more than two the number of rats that shall have been assigned to make up any other group. For a given sample, no less than 7 rats should be used, whereas 10 or more rats per given assay group will reduce the error due to variation.

When the assembling of all groups shall have been completed, the total number of rats in each group shall be the same. Not more than 3 rats from one litter shall be assigned to the assay group unless an equal number of rats from the same litter is assigned to the reference group.

E. ASSAY PERIOD

A rat shall be suitable for the assay period, provided the depletion period shall have exceeded 18 days and shall not have exceeded 25 days, and provided the said rat shall manifest evidence of rickets. The presence of rickets may be established by examination of a leg bone of one member of a litter by the "line test" described below, by palpation, or by x-ray examination of the animals selected for assay.

Rats suitable for the assay period shall be weighed and segregated in individual cages provided with screen bottoms and shall be provided with the rachitogenic diet and distilled water *ad libitum*. On any calendar day of the assay period, the assay and reference groups shall receive a rachitogenic diet compounded from the same lot of ingredients.

The assay period shall be seven 24 hour days in length. Throughout the first 6 days of the assay period, each rat in any one assay group shall be fed daily the calculated dose of vitamin D milk, and throughout the first 6 days of the assay period each rat in any one reference group shall be fed daily a dose of reference oil,* except that the following deviation from the daily feeding shall be permissible: that the daily dose of milk or reference oil may be doubled on the day preceding a one day holiday falling within the first 6 days of the assay period. (See *J. Assoc. Official Agr. Chem.*, 21:90, 1938, for additional feeding options.) As soon as possible after the termination of the assay period, each rat shall be killed by suitable means and one or more leg bones examined for healing of the rachitic metaphyses, according to the line test described below.

The reference oil shall be diluted before feeding with an edible vegetable oil free from vitamins A and D. The diluted oil shall be stored in the dark at a temperature not exceeding 50° F., and the duration of this storage shall not exceed 30 days. Not more

* There is no unanimity of opinion as to whether it is necessary, from the practical standpoint, to feed skim milk solids at all assay levels, and, therefore, further experimental work must be done before a recommendation can be made with regard to the feeding of the solids to the reference oil group

than 0.1 ml. of the diluted reference oil shall be fed as a daily dose, except as provided for above. Inasmuch as the dose of the reference oil required to give a standard narrow continuous line of healing may vary from laboratory to laboratory, depending upon conditions, the dose of reference oil to be fed shall be determined prior to the running of routine assays. By way of orientation, it may be said that a supplement falling between $\frac{1}{2}$ and $\frac{2}{3}$ U. S. P. unit of standard per day (U.S.P. reference oil) will be found to produce a narrow continuous line of calcium deposits in approximately three-fourths of the test animals. It is advisable to use the minimum amount of U. S. P. reference oil required to produce this degree of healing for comparison. The reference oil shall be diluted so that one-sixth of the dose necessary to produce positive macroscopic evidence of calcification shall be administered each day for the first 6 days of the assay period.

In the reference group already mentioned, U. S. P. reference cod liver oil shall be used as a comparative standard for the vitamin D assay milk.

F. PREPARATION OF STANDARD TO GIVE $\frac{1}{2}$ TO $\frac{2}{3}$ U. S. P. UNITS

I. SAMPLE CALCULATION

The U. S. P. reference cod liver oil now in use contains 95 U. S. P. units per gm.; 1 unit is contained in 10.52 mg. In making up each ml. of diluted oil use 5.26 mg. of the reference oil, and each 0.1 ml. of the diluted oil will contain $\frac{1}{2}$ unit of vitamin D. Other concentrations of the diluted oil can be calculated in a similar manner. From the amount of U. S. P. reference oil determined to be necessary to produce positive macroscopic evidence of calcification, the calculated amount of milk, based on the unitage claimed, is fed during the first 6 days in six equal doses. If $\frac{1}{2}$ unit per day for 6 days (3.0 U. S. P. or 31.56 mg.) is necessary to produce the type of calcification above described, then the dose of vitamin D milk which would be expected to produce a degree of calcification equal to or greater than the degree of calcification obtained in the reference group may be calculated.

For a vitamin D milk carrying 200 U. S. P. units per quart, this amounts to $\frac{3}{200}$ of 946 ml. in one quart, or 14.14 ml. total dose; if fed in graded doses during the first 6 days, $14.14 \div 6 = 2.35$ ml. daily. A vitamin D milk carrying 400 U. S. P. units per quart would require just one-half, or a total dose of 7.07 ml. administered at a level of 1.18 ml. for 6 days. If a higher increment of U. S. P. reference oil is required, then the amount of milk to be fed, based on unitage claimed, is calculated. In the method just described, the milk shall be fed in small dishes separate and apart from the diet, and the reference oil may be fed in the same manner or directly to the rat by calibrated dropper or pipette.

Certain variations from this method, such as the length of the assay period and the manner in which the milk and reference oil are fed, may be used without influencing the validity of the results obtained.

Explanatory Note: Experimental trials and the experience of those now conducting routine assays of vitamin D milk show that departures from the U. S. P. XI method may be made in the manner in which the milk sample and the reference oil are fed, and in the length of the assay period.

If it is necessary to feed a relatively large quantity of milk, a single feeding may not be possible, and daily feedings for a period of days are necessary.

The extent of calcification at the end of a 7 day assay period is not as marked as at the end of a 10 day period. Hence it may be necessary to feed higher levels of milk and reference oil to obtain an adequate response at the end of a 7 day period.

As has been indicated, if use is made of any of the alternatives described, the dose of reference oil and milk will have to be calculated according to the chosen option. In any event, both the U. S. P. reference oil and the milk sample must be fed according to the same plan.

G. LINE TEST

The effect of the milk feeding and response to the U. S. P. reference oil is determined as follows: The line test shall be made on the proximal end of the tibiae or distal end of the radii or ulnae. The end of the desired bone is removed from the animal and cleaned of adhering tissue. A longitudinal median section shall be made through the end of the bone with a clean sharp blade to expose a plane surface through the junction of the epiphysis and diaphysis. In one assay, the same bone of all the animals must be used and sectioned through the same plane. Both sections of the bone shall be rinsed in distilled water and shall then be immersed in a freshly prepared 2 per cent aqueous solution of silver nitrate for 1 minute. The sections shall then be rinsed in distilled water, and the sectioned surfaces of the bone shall be exposed in water to daylight or other source of actinic light until the calcified areas have developed a clearly defined stain without marked discoloration of the uncalcified area. Evidence of congestion in the rachitic metaphyses should be clearly distinguished from calcium salts stained with silver, as the principal criterion of healing is the development of the line at the provisional zone of calcification.

As an optional procedure, the bone after being removed and sectioned may be placed in 10 per cent formaldehyde or 95 per cent alcohol for a period of 3 to 4 hours, after which it is rinsed in distilled water and stained with silver nitrate in the manner previously described. The use of this modification is purely optional; however, the bone specimens seem to stain more distinctly, the tissues having been hardened and cleared. The time that the specimen is resident in the formaldehyde is too short to cause any significant decalcification due to formic acid. If longer periods of storage are used, 95 per cent alcohol is preferable. Other modifications of the staining technic may be used, providing they are equally satisfactory in showing calcified areas.

H. RECORDING OF DATA

Records shall be made, immediately after staining, of the extent and degree of calcification of the rachitic metaphyses of every section. Numerical values shall be assigned to the extent and degree of calcification of the rachitic metaphyses of the bones examined by the line test, so that it will be possible to average the performance of the group. On the day beginning the assay period and on the 7th day thereafter, a record shall be made of the body weight of each rat.

I. VITAMIN D POTENCY OF THE MILK

In determining the vitamin D potency of the milk, the average performance of the reference oil group with respect to the healing of the rachitic metaphysis shall be such that two-thirds or more, but not less than 7 of the animals of this group, show macroscopic evidence of calcification. When the average response of the assay group is equal to or greater than that of the reference oil group, the vitamin D content of the milk fed during the assay period is equal to or greater than the vitamin D content of the reference oil fed during the assay period. When the average response of the assay group is less than that of the reference group, the vitamin D content of the milk fed during the assay period is less than the vitamin D content of the reference oil during the assay period. The data from a rat shall be considered valid for establishing the average performance of a group only on the condition that the weight of the rat at the termination of the assay period shall equal or exceed the weight of the rat at the beginning day of the assay period, and on the condition that the rat has consumed each prescribed dose of milk within 24 hours from the time it was fed.

The vitamin D potency of the milk is then calculated as follows:

I. CALCULATION OF POTENCY

The vitamin D potency of the milk may be calculated by using the following formula:

$$Y \times \frac{946}{X} = \text{U. S. P. units per qt.}$$

Y = U. S. P. units of vitamin D (total dose) necessary to produce a narrow line of calcification in the reference groups.

946 is the ml. in 1 qt.

X is the ml. of milk (total dose) necessary to produce in the assay group an equal or greater degree of calcification than that produced in the reference group.

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PART IV. CHEMICAL METHODS

Association of Official Agricultural Chemists

The methods here described are those adopted by the Association of Official Agricultural Chemists and are quoted with the permission of the Board of Editors of that Association.

Strengths of acids and ammonia, when not otherwise specified, are those of the ordinary concentrated reagents. In the expressions $(1 + 2)$, $(5 + 4)$, etc., used in connection with the name of a reagent the first numeral indicates the volume of reagent and the second the volume of water.

All temperatures, when not otherwise specified, are expressed in degrees Centigrade and the usual abbreviation "C." is omitted.

A. MILK

I. COLLECTION OF SAMPLE — OFFICIAL

The quantity of sample required depends upon the number of determinations to be made. For the usual analysis collect 250–500 ml. ($\frac{1}{2}$ –1 pint) of sample; for the fat determination only, 50–60 ml. (approximately 2 fl. oz.) will suffice.

In the case of bottled milk, collect one or more bottles as prepared for sale. In sampling bulk milk thoroughly mix by pouring from one clean vessel into another three or four times. If this procedure is impracticable, thoroughly stir the milk for at least 30 seconds with a suitable appliance long enough to reach to the bottom of the container. If cream has formed on the milk, continue the mixing until all cream is detached from the sides of the vessel and evenly emulsified throughout the liquid.

Place the samples in non-absorbent, airtight containers and keep them in the cold, but at a temperature above freezing, until ready for examination. When transported by mail, express, or otherwise, completely fill the containers, tightly stopper, and mark for identification. A suitable quantity of preservative (HgCl_2 , $\text{K}_2\text{Cr}_2\text{O}_7$, or HCHO) may be used unless the presence of preservative is objectionable in connection with physical or chemical tests to be applied in addition to the determination of fat.

II. PREPARATION OF SAMPLE — OFFICIAL

Before withdrawing portions for analytical determinations, bring the sample to a temperature of 15–20° and mix thoroughly by pouring into a clean receptacle and back, until a homogeneous mixture is assured. If lumps of cream do not completely disappear, warm the sample to about 38°, mix thoroughly, then cool to 15–20°. In case a measured volume is required in a determination, bring the temperature of the sample to 20° before pipetting.

III. SPECIFIC GRAVITY — TENTATIVE

Determine specific gravity at 15.6/15.6° by means of a pycnometer or a standard hydrometer.

IV. ACIDITY — TENTATIVE

Dilute 10–20 ml. of the milk with an equal volume of recently boiled and cooled H_2O and titrate with standard NaOH solution, using phenolphthalein indicator (1 gm. phenolphthalein in 100 ml. of 95 per cent alcohol). Express the result as percentage of lactic acid. The determination may be conveniently made by measuring 17.6 ml. of the prepared sample with the 17.6 ml. Babcock pipette, diluting with an equal volume of recently boiled and cooled H_2O , washing out the pipette with CO_2 -free H_2O , and titrating with 0.1 N NaOH solution, using 0.5 ml. of phenolphthalein indicator. The number of ml. of 0.1 N NaOH solution required divided by 20 gives the percentage of lactic acid.

V. CITRIC ACID¹ — TENTATIVE

1. PREPARATION OF SAMPLE

To 50 gm. of milk in a 150 ml. beaker, add about 100 mg. of tartaric acid and 6 ml. of normal H_2SO_4 and place on the steam bath for 15 minutes. Immediately add 3 ml. of a 20 per cent phosphotungstic acid solution, mix well, and return to the steam bath for 5 minutes. Transfer to a 250 ml. volumetric flask with 95 per cent alcohol, cool, dilute to the mark with the alcohol, mix, and filter through a folded paper. Pipette 200 ml. of the clear filtrate into a centrifuge bottle.

2. REAGENTS

Potassium bromide solution—Dissolve 15 gm. of KBr in 40 ml. of H_2O .

Potassium permanganate solution—Dissolve 5 gm. of KMnO_4 in H_2O and dilute to 100 ml.

Ferrous sulfate solution—Dissolve 40 gm. of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ in 100 ml. of H_2O containing 1 ml. of H_2SO_4 .

Lead acetate solution—Dissolve 75 gm. of normal Pb acetate in H_2O , add 1 ml. of glacial acetic acid, and dilute to 250 ml.

3. DETERMINATION

To the solution in the centrifuge bottle, add 10 ml. of the Pb acetate solution, shake vigorously for about 2 minutes, and centrifuge at about 1,000 r.p.m. for 15 minutes. Carefully decant the supernatant liquid from the precipitated Pb salts and test with a small quantity of the Pb solution. If a precipitate forms, return to the centrifuge bottle, add more Pb solution, shake, and again centrifuge. If the sediment lifts, repeat the centrifuging, increasing the speed and time. Allow the bottle to drain thoroughly by inverting it for several minutes. To the Pb salts in the centrifuge bottle add about 150 ml. of H_2O , shake thoroughly, and pass in H_2S to saturation. Transfer to a 250 ml. volumetric flask, dilute with H_2O to mark, mix, and filter through a folded paper. Pipette 200 ml. of the filtrate into a 500 ml. Erlenmeyer flask, and evaporate to about 75 ml. Cool, and add 10 ml. of H_2SO_4 (1 + 1) and 5 ml. of the KBr solution. Heat the mixture to $48\text{--}50^\circ$, allow to stand for 5 minutes, and add 50 ml. of the KMnO_4 solution. Mix, and allow to stand 1 minute. Stopper the flask, shake for about 1 minute, and allow to stand 3 minutes. (During this time there should be a heavy deposit of MnO_2 ; if necessary, add more KMnO_4 to assure an excess of the oxidizing agent. If at any time during the oxidation the precipitated MnO_2 disappears, discard the determination and repeat, using more KMnO_4 .) Remove the MnO_2 with the FeSO_4 solution (about 20 ml.), cool to about 15° , stopper the flask, shake vigorously for several minutes, and place in the refrigerator overnight. Decant the supernatant liquid onto a thin, tightly tamped pad of asbestos in a Gooch crucible (it is important that filtration be completed as quickly as possible). Note volume of filtrate (S in the formula) and use the filtrate to transfer the precipitate to the crucible. Wash the contents of the crucible at once with 50 ml. of ice cold H_2O . Dry by aspirating with dry air or in a vacuum desiccator and weigh.

Remove the pentabromacetone by treating the contents of the crucible with three portions of 20 ml. each of alcohol and three portions of 20 ml. each of ether. Again dry and weigh. The difference in the two weights represents the weight of pentabromacetone.

Calculate the mg. of citric acid in the portion taken for analysis by the following formula:

$$X = 0.695P + 0.028S, \text{ in which}$$

X = mg. of citric acid in the portion taken for analysis,
 P = weight of pentabromacetone in milligrams; and
 S = volume of filtrate (ml.).

VI. TOTAL SOLIDS

1. METHOD I—OFFICIAL

Weigh a flat-bottomed dish of not less than 5 cm. diameter. If desired, the dish may have spread in it, prior to weighing, 15–20 gm. of pure dry sand. Pipette into the dish 1.5–3 ml. of the sample, weigh quickly, and heat on steam bath 30 minutes, then in oven (preferably vacuum) at the temperature of boiling H_2O to constant weight. Cool in a desiccator, and then weigh quickly to avoid the absorption of moisture.

2. METHOD II—TENTATIVE

(Approximate Total Solids)

Calculate the total solids by means of the formula $S = 0.25 L + 1.2 F$, in which S = total solids, L = the lactometer reading at 15.6° (sp. gr. as determined in III times 1,000 minus 1,000), and F = the percentage of fat in the milk.

VII. ASH — OFFICIAL

Into a weighed dish pipette about 20 ml. of the prepared sample, weigh quickly, add 6 ml. of HNO_3 , evaporate to dryness, and ignite at a temperature below redness until the ash is free from C. Cool in a desiccator, weigh, and report the increase in weight as ash.

VIII. TOTAL NITROGEN — OFFICIAL

Transfer 5 gm. of the sample to a Kjeldahl digestion flask. Add approximately 0.7 gm. of HgO , or its equivalent in metallic Hg ,*

* In place of the Hg or HgO , 1 gm. of crystallized $CuSO_4 \cdot 5 H_2O$ may be substituted.

15–18 gm. of K_2SO_4 or anhydrous Na_2SO_4 and 25 ml. of concentrated H_2SO_4 . Heat the mixture gently until frothing ceases, then boil briskly and continue the digestion for a time after the mixture is colorless or nearly so, or until oxidation is complete (approximately 2 hours). Cool, add about 200 ml. of H_2O , a few pieces of granulated Zn or pumice stone to prevent bumping, and, with shaking, 50 ml. of a solution of K_2S (40 gm. of commercial K_2S in 1 liter of H_2O), or of Na_2S (40 gm. of commercial Na_2S in 1 liter of H_2O), or of $Na_2S_2O_3$ (80 gm. of $Na_2S_2O_3 \cdot 5H_2O$ in 1 liter of H_2O). (If $Na_2S_2O_3$ is to be used, it should first be mixed with the NaOH so that they may be added together. When no Hg or HgO is used the addition of K_2S or Na_2S or $Na_2S_2O_3$ solution is unnecessary.) Next add sufficient NaOH solution (450 gm. of commercial NaOH, free from nitrates, in 1 liter of H_2O) to make the reaction strongly alkaline (50 ml. is usually sufficient), pouring it down the side of the flask so that it does not mix at once with the acid solution. Connect the flask to the condenser by means of a Kjeldahl connecting bulb, taking care that the tip of the condenser extends below the surface of the standard acid in the receiver; mix the contents by shaking and distill until all NH_3 has passed over into a measured quantity of standardized 0.1 N HCl or H_2SO_4 . (The first 150 ml. of the distillate will generally contain all the NH_3 .) Titrate with standardized 0.1 N alkali solution, using methyl red or cochineal indicator. Multiply the percentage of N by 6.38 to obtain the equivalent percentage of milk proteins.

(Previous to use, the reagents should be tested by a blank experiment with sugar. The sugar partially reduces any nitrates present that might otherwise escape notice.)

IX. CASEIN

(This determination should be made while the milk is fresh, or nearly so. If it is not possible to make this determination within 24 hours, add 1 part of HCHO to 2,500 parts of milk and keep in a cool place.)

1. METHOD I—OFFICIAL

Place 10 gm. of the sample in a beaker with 90 ml. of H_2O at 40–42° and add at once 1.5 ml. of acetic acid (1 + 9). Stir, and let stand 3–5 minutes. Decant on an acid-washed filter, wash by

decantation two or three times with cold H_2O , and transfer the precipitate to the filter. Wash once or twice on the filter. The filtrate should be clear, or very nearly so. If the first portions of the filtrate are not clear, repeat the filtration, and complete the washing of the precipitate. Determine N in the washed precipitate and filter paper as directed under VIII, and multiply by 6.38 to obtain the equivalent of casein.

To a sample of milk that has been preserved, the acetic acid should be added in small portions, a few drops at a time, with stirring, and the addition should be continued until the liquid above the precipitate becomes clear, or very nearly so.

2. METHOD II²—TENTATIVE

A. REAGENT

Pipette 250 ml. of N acetic acid into a 1,000 ml. flask. Add 125 ml. of normal CO_2 -free NaOH. Make up to 1,000 ml. with CO_2 -free distilled H_2O and mix thoroughly.

B. DETERMINATION

Pipette 20 ml. of the sample into a 100 ml. flask. Add 50 ml. of the reagent, mix, make up to volume with distilled H_2O , and shake well. Set the flask in hot H_2O (50 – 60° , not over 60°) and let stand 15 minutes. Cool to room temperature, add 0.5 gm. of celite analytical filter aid, shake thoroughly and filter clear through a suitable folded paper, taking care to prevent evaporation during filtration. Determine N (A) in 50 ml. of the clear filtrate, and determine total N (B) in 10 ml. of milk. Multiply (B — A) by 6.38. This gives the casein in 10 ml. of the milk. Report grams of casein per 100 ml. of milk, or divide the grams per 100 ml. by the density of the milk and report as percentage by weight.

X. ALBUMIN—OFFICIAL

Exactly neutralize the filtrate obtained under IX, Method I, with 10 per cent NaOH solution, add 0.3 ml. of acetic acid (1 + 9), and heat on a steam bath until the albumin is completely precipitated. Collect the precipitate on an acid-washed filter, wash with cold H_2O , determine the nitrogen as directed under VIII, and multiply by 6.38 to obtain the equivalent of albumin.

XI. LACTOSE

1. OPTICAL METHOD—OFFICIAL

A. REAGENTS

a. *Acid mercuric nitrate solution*—Dissolve Hg in twice its weight of HNO_3 and dilute with a fivefold volume of H_2O .

b. *Mercuric iodide solution*³—Dissolve 33.2 gm. of KI and 13.5 gm. of HgCl_2 in 200 ml. of glacial acetic acid and 640 ml. of H_2O .

B. DETERMINATION

Determine the specific gravity of the milk as directed under III. The quantity of sample to be taken for the determination varies with

TABLE I

VOLUMES OF MILK CORRESPONDING TO A LACTOSE DOUBLE NORMAL WEIGHT⁴

Specific gravity of milk	Volume of milk for a lactose double normal weight (Ventzke Scale)	Specific gravity of milk	Volume of milk for a lactose double normal weight (Ventzke Scale)
	ml.		ml.
1.024	64.25	1.030	63.90
1.025	64.20	1.031	63.80
1.026	64.15	1.032	63.75
1.027	64.05	1.033	63.70
1.028	64.00	1.034	63.65
1.029	63.95	1.035	63.55
		1.036	63.50

the specific gravity and is to be measured at the same temperature at which the specific gravity is taken. The volume to be measured will be found in Table I, which is based upon twice the normal weight of lactose (32.9 gm. per 100 ml.) for the Ventzke sugar scale.

Place the quantity of milk indicated in Table I in a flask graduated at 102.6 ml. Add 18–20 ml. of the acid $\text{Hg}(\text{NO}_3)_2$ solution or 30 ml. of the HgI_2 solution, followed by enough 5 per cent phosphotungstic acid solution to make to the mark, shake frequently for at least 15 minutes, filter through a dry filter, and polarize. It is not necessary to heat before polarizing. If a 200 mm. tube is used, divide the polariscope reading by 2 (or, if a 400 mm. tube is used, by 4) to obtain the percentage of lactose in the sample.

2. GRAVIMETRIC METHOD—OFFICIAL

Dilute 25 gm. of the sample with 400 ml. of H_2O in a 500 ml. volumetric flask, add 10 ml. of CuSO_4 solution (34.639 gm. of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, dissolved in H_2O , diluted to 500 ml. and filtered through an asbestos mat) and about 7.5 ml. of a KOH solution of such strength that one volume is just sufficient to precipitate completely the Cu as hydroxide from one volume of the CuSO_4 solution. (Instead of KOH solution of this strength, 8.8 ml. of 0.5 N NaOH solution may be used.) After the addition of the alkali solution the mixture must still have an acid reaction and contain Cu in solution. Fill the flask to the 500 ml. mark, mix, filter through a dry filter, and determine lactose in an aliquot of the filtrate by precipitation of Cu_2O from Fehling's Solution. Details of the method are given in Methods of Analysis, A.O.A.C., 1940, p. 500.

XII. FAT

1. ROESE-GOTTLIEB METHOD^b—OFFICIAL

Transfer 10 gm. of the sample to a Röhrig tube or a similar apparatus, add 1.25 ml. of NH_4OH (2 ml. if the sample is sour), and mix thoroughly. Add 10 ml. of 95 per cent alcohol and mix well. Add 25 ml. of ether, shake vigorously for 30 seconds, add 25 ml. of petroleum benzin (redistilled slowly at a temperature below 65°), and shake again for 30 seconds. Let stand 20 minutes, or until the upper liquid is practically clear. Draw off as much as possible of the ether-fat solution (usually 0.5–0.8 ml. will be left) into a flask through a small, quick-acting filter. Again extract the liquid remaining in the tube, this time with 15 ml. of each solvent; shake vigorously 30 seconds after each addition and allow to settle. Draw off the clear solution through the small filter into the same flask as before and wash the tip of the spigot, the funnel, and the filter with a few ml. of a mixture of the two solvents, in equal parts, free from suspended H_2O . To insure complete removal of the fat, a third extraction is necessary. (This third extraction yields less than 1 mg. of fat if the previous solutions have been drawn off closely.) Add a glass bead and evaporate the ethers slowly on a warm surface; then dry the fat in a boiling water oven to constant weight. Weigh the flask with a similar flask as a counterpoise. Do not wipe the flask immediately before weighing.

Remove the fat completely with petroleum benzin. Deduct the weight of the dried flask with residue and bead to obtain the weight of fat. Finally, correct this weight by a blank determination on the reagents used.

2. BABCOCK METHOD⁶—OFFICIAL

A. REAGENT

Sulfuric acid—Sp. gr. 1.82–1.83 at 20°.

B. APPARATUS

a. *Standard Babcock test bottle for milk*—8 per cent, 18 gm., 6 in. milk test bottle, total height 150–165 mm. (5.9–6.5 in.).

The bottom of the bottle shall be flat, and the axis of the neck shall be vertical when the bottle stands on a level surface. The charge of milk for the bottle shall be 18 gm.

Bulb—The capacity of the bulb to the junction with the neck shall be not less than 45 ml. The shape of the bulb shall be either cylindrical or conical. If cylindrical, the outside diameter shall be between 34 and 36 mm.; if conical, the outside diameter of the base shall be between 31 and 33 mm., and the maximum diameter between 35 and 37 mm.

Neck—The neck shall be cylindrical and of uniform diameter from at least 5 mm. below the lowest graduation mark to at least 5 mm. above the highest. The top of the neck shall be flared to a diameter of not less than 10 mm. The graduated portion of the neck shall have a length of not less than 63.5 mm. The total per cent graduation shall be 8. The graduations shall represent whole per cent, 0.5 per cent, and 0.1 per cent, respectively, from 0.0 to 8.0 per cent. The tenths per cent graduations shall be not less than 3 mm. in length; the 0.5 per cent graduations shall be not less than 4 mm. in length and shall project 1 mm. to the left; and the whole per cent graduations shall extend at least half-way around the neck to the right and shall project at least 2 mm. to the left of the tenths per cent graduations. Each whole per cent graduation shall be numbered, the number being placed to the left of the scale. The capacity of the neck for each whole per cent on the scale shall be 0.20 ml. The maximum error of the total graduation or any part thereof shall not exceed the volume of the smallest unit of the graduation.

Each bottle shall be so constructed as to withstand the stress to which it will be subjected in the centrifuge.

a (1). *Testing*—The Hg and cork, alcohol and burette, and alcohol and brass plunger methods may be employed for the rapid testing of the bottles, but the accuracy of any questionable bottle shall be determined by calibration with Hg (13.5471 gm. of clean, dry Hg at 20° to be equal to 5 per cent on the scale of an 18 gm. bottle and 10 per cent on the scale of a 9 gm. bottle), the bottle being previously filled to zero with Hg.

b. *Pipette*—The standard milk pipette shall conform to the following specifications:

Total length, not more than 330 mm.

Outside diameter of suction tube, 6–8 mm.

Length of suction tube, 130 mm.

Outside diameter of delivery tube, 4.5–5.5 mm.

Length of delivery tube, 100–120 mm.

Distance of graduation mark above bulb, 15–45 mm.

Nozzle, straight.

Graduation, to contain 17.6 ml. of H₂O at 20° when the bottom of the meniscus coincides with the mark on the suction tube.

Delivery in 5–8 seconds.

The maximum error in the graduation shall not exceed 0.05 ml.

The pipette is to be marked "Holds 17.6 ml."

b (1). *Testing*—The pipette shall be tested by measuring from a burette the volume of H₂O (at 20°) which it holds up to the graduation mark.

c. *Acid measure*—The device employed to measure H₂SO₄, whether a graduated cylinder or a pipette attached to a Swedish acid bottle, shall be graduated to deliver 17.5 ml.

d. *Centrifuge or "tester"*—The standard centrifuge, however driven, shall be constructed throughout and so mounted as to be capable, when filled to capacity, of rotating at the necessary speed with a minimum of vibration and without liability of causing injury or accident. It shall be heated, electrically or otherwise, to a temperature of at least 55° during the process of centrifuging. It shall be provided with a speed indicator, permanently attached, if possible. The proper rate of rotation may be ascertained by reference to the table below. By "diameter of wheel" is meant the distance between the inside bottoms of opposite cups measured through the center of

rotation of the centrifuge wheel while the cups are horizontally extended:

Diameter of wheel, in inches	10	12	14	16	18	20	22	24
No. revolutions per minute...	1,074	980	909	848	800	759	724	693

e. *Dividers or calipers*—For measuring the fat column.

f. *Water bath for test bottles*—Provided with a thermometer and a device for maintaining a temperature of 55–60°.

C. DETERMINATION

Transfer 18 gm. of the sample, prepared as directed under II, to the milk-test bottle by means of the pipette. Blow out the milk remaining in the pipette tip after free outflow has ceased. Add 17.5 ml. of H_2SO_4 , preferably not all at one time, pouring it down the side of the neck of the bottle in such a way as to wash all traces of the milk into the bulb. The temperature of the acid shall be about 15–20°. Shake until all traces of curd have disappeared; then transfer the bottle to the centrifuge; counter-balance it; and, after the proper speed has been attained, whirl 5 minutes. Add soft H_2O at 60°, or above, until the bulb of the bottle is filled. Whirl 2 minutes. Add hot H_2O until the liquid column approaches the top graduation of the scale. Whirl 1 minute longer at a temperature of 55–60°. Transfer the bottle to the warm water bath maintained at a temperature of 55–60°, immerse it to the level of the top of the fat column, and leave it there until the column is in equilibrium and the lower fat surface has assumed a final form. Remove the bottle from the bath; wipe it; and, with the aid of dividers or calipers, measure the fat column, in terms of percentage by weight, from its lower surface to the highest point of the upper meniscus.

The fat column, at the time of measurement, should be translucent, of a golden yellow or amber color, and free from visible suspended particles. Reject all tests in which the fat column is milky or shows the presence of curd or of charred matter, or in which the reading is indistinct or uncertain.

XIII. ADDED WATER

1. ACETIC SERUM METHOD⁷—OFFICIAL

a. *Zeiss immersion refractometer reading*—To 100 ml. of the milk, measured at 20° into a beaker, add 2 ml. of 25 per cent acetic

acid (sp. gr. 1.035). Cover the beaker with a watch glass and place in a water bath at 70° for 20 minutes. Place the beaker in ice H_2O for 10 minutes and separate the curd from the serum by rapid filtration through a small filter. Transfer a portion of the clear serum to a refractometer beaker, place in the constant temperature bath, and take the refractometer reading when the temperature of the serum has been brought to exactly 20° , as determined by a thermometer graduated in tenths of a degree. A reading below 39 indicates added H_2O ; between 39 and 40, the addition of H_2O is suspected. When the reading is 40 or below, determine the ash in the serum as directed under (b).

b. *Ash*—Transfer 25 ml. of the serum to a weighed flat-bottomed platinum dish and evaporate to dryness on a water bath. Heat over a low flame (to avoid spattering) until the contents are thoroughly charred, place the dish in an electric muffle, preferably with pyrometer attached, and ignite to a white ash at a temperature not greater than 500° . Cool and weigh. Express the result as grams per 100 ml. A result below 0.715 gm. per 100 ml. indicates added H_2O . The acetic serum ash, multiplied by the factor 1.021, equals the sour serum ash (dilution of the acetic serum being 2 per cent).

2. SOUR SERUM METHOD—OFFICIAL

a. *Zeiss immersion refractometer reading*⁸—Allow the milk to become completely sour, filter, and determine the immersion refractometer reading of the clear serum at 20° . A reading below 38.3 indicates added H_2O .

b. *Ash*⁹—Determine the ash in 25 ml. of the serum, obtained in a, as directed under XIII, 1, b. A result below 0.730 gm. per 100 ml. indicates added H_2O .

3. COPPER SERUM METHOD¹⁰—OFFICIAL

To 1 volume of $CuSO_4$ solution (72.5 gm. of $CuSO_4 \cdot 5H_2O$ per liter, adjusted if necessary to read 36 at 20° on the scale of the Zeiss immersion refractometer, or to a specific gravity of 1.0443 at $20^{\circ}/4^{\circ}$), add four volumes of milk. Shake well and filter. Determine the refractometer reading of the clear serum at 20° . A reading below 36 indicates added H_2O . When the refractometer reading is 36 or below, determine the ash of the sour serum as directed under XIII, 2, b or of the acetic serum as directed under XIII, 1, b, above.

4. CRYOSCOPIC METHOD¹¹—OFFICIALA. APPARATUS
(FIGURE XXXII)

a. *Cryoscope* — A cylindrical-shaped Dewar flask of 1 liter capacity and 28 cm. internal depth, surrounded by a metal casing, is tightly closed by means of a large cork of about 3 cm. thickness. Through the center of the cork is tightly fitted a medium thin-walled glass or metal tube, 250 mm. in length by 33 mm. outside diameter. At one side of the cork is inserted a narrow metal inlet tube, the lower end of which is formed into a perforated loop near the bottom of the flask. At the opposite side is a metal tube of T-shape construction and 6 mm. internal diameter, intended to afford escape for

vapors, and also for introducing volatile fluid into the apparatus. At the back portion of the cork is fitted a control thermometer, the bulb of which extends nearly to the bottom of the flask. The freezing test tube is of thin glass, about 240 mm. in length by 29 mm. outside diameter, and fits closely into the larger tube, which is sealed into the cork. In the rubber stopper of the freezing tube is fitted the standard thermometer. The thermometer is constructed of such length as to enable insertion of the bulb nearly to the bottom of the tube and at the same time allow complete exposure of the scale above the stopper. At the right side of the thermometer a stirring device made of non-corrodible low conductivity metal is fitted into the stopper through a short section of thin-walled metal tubing. The

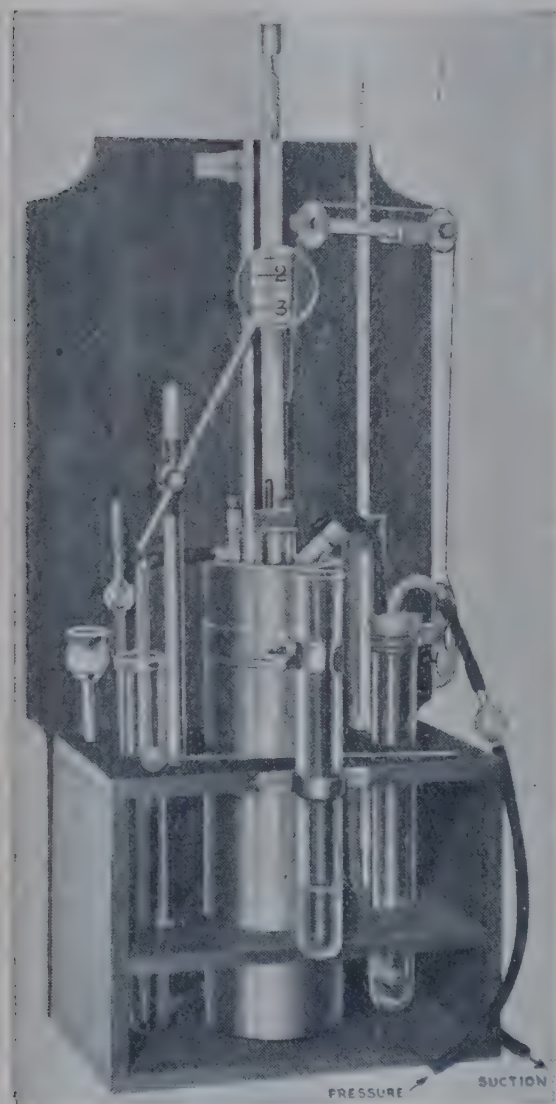


FIGURE XXXII—The Hortvet Cryoscope

lower end extends nearly to the bottom of the test tube and is provided with a horizontal loop encircling the thermometer. At the left of the thermometer is a freezing-starter attachment inserted through an opening in the stopper formed by means of a short section of metal tubing. This device consists of a non-corrodible metal rod, at the lower end of which is a 10 mm. length opening for the purpose of carrying a small fragment of ice. At one side of the cryoscope is installed an air-drying arrangement which consists of a Folin absorption bulb inserted through a tightly fitting stopper and extending nearly to the bottom of a large-sized test tube. A short section of glass tubing is inserted through a second opening in the stopper and is connected to the vaporizing tube which enters the cryoscope. Sulfuric acid is poured into the drying tube to a level slightly above the small inner bulb. At the opposite side of the apparatus is arranged a drain tube for the purpose of conducting vapors away from the operator. By means of a pressure and suction pump, dry air may be forced into the apparatus at a suitable rate and the mixed vapors conducted out through the base of the drain tube into the sink. An adjustable lens is mounted in a convenient position in front of the thermometer for the purpose of magnifying the scale.

b. *Standard thermometer*—The standard thermometer designed especially for testing milk is a solid-stem instrument measuring a total length of 58 cm., with a scale portion measuring about 30 cm. The total scale range is 3° , from $+1^{\circ}$ to -2° , and each degree division is subdivided into tenths and hundredths. The length of a degree division approximates one decimeter, thus making the smallest subdivisions of such magnitudes as to enable easy observation and readings estimated to 0.001° . Standardize the thermometer as directed under B. Check at frequent intervals, once a week or as often as may be necessary, to keep an accurate record of any changes that may occur.

c. *Control thermometer*—A solid-stem instrument approximately 58 cm. in length and having a scale range of $+20^{\circ}$ to -30° . Test in a bath of melting crushed ice for the purpose of determining whether the 0-mark on the scale is correct. The scale graduations should be accurate to within 0.10° .

B. STANDARDIZATION OF THE THERMOMETER

Make three freezing-point determinations by the procedure given under C on each of the following:

a. *Recently boiled distilled water.*

b. *Sucrose solution*—Dissolve 7 gm. of pure sucrose in H_2O and make the solution to a volume of 100 ml. at 20° .

c. *Sucrose solution*—Dissolve 10 gm. of pure sucrose in H_2O and make the solution to a volume of 100 ml. at 20° .

(A sample of pure sucrose may be obtained by application to the Director of the Bureau of Standards, Department of Commerce, Washington, D. C.)

Tabulate the results in the following form:

Express the results as degrees freezing-point depression below the average of the observed freezing points obtained on the sample of pure H_2O ($\pm W$), which may be above (+) or below (—) the 0-mark on the scale.

Freezing Point Observations	Pure Water	7 Grams Sucrose Solution		10 Grams Sucrose Solution	
		Observed freezing point (—S)	Freezing-point depression S—W (algebraic)	Observed freezing point (—S)	Freezing-point depression S—W (algebraic)
1st					
2nd					
3rd					
Averages	$\pm W$	xxxxxxx		xxxxxxx	

Obtain each freezing-point depression of the sucrose solutions by the algebraic subtraction of the average of the freezing-point readings of pure H_2O ($\pm W$) from each observed freezing point.

Omit adventitious results, *i.e.*, results which are in marked disagreement with other results obtained by carefully following instructions.

Apply the average of the freezing-point depressions obtained on the standard sucrose solutions for the purpose of correcting the thermometer readings obtained on samples of milk in the manner illustrated in the tables accompanying Figure XXXIII.

C. DETERMINATION

(Make freezing-point determinations only on samples of milk that show an acidity of not more than 0.18 per cent when determined as directed under IV.)

Insert the funnel-tube into the vertical portion of the T-tube at one side of the apparatus and pour in 400 ml. of ether previously cooled to 10° or lower. Close the vertical tube by means of a small cork and connect the pressure pump to the inlet tube of the air-drying attachment. Adjust the pump so as to pass air through the apparatus at a moderate rate, as may be judged by the agitation of the H_2SO_4 in the drying tube. Continuous vaporization of the ether will cause a lowering of the temperature in the flask, from ordinary room temperature to 0° in from 5 to 10 minutes. Continue the temperature lowering until the control thermometer registers near -3° . At this stage, by lowering the gage tube into the ether bath, then closing the top by means of the forefinger and raising to a suitable height, an estimate can be made as to the quantity of ether necessary to pour in for the purpose of restoring the 400 ml. volume. When the volume of ether has been adjusted to 400 ml., an additional 10–15 ml. is sufficient on an average for each succeeding determination. Pour into the freezing test tube sufficient H_2O (30–35 ml.), boiled and cooled to 10° or lower, to submerge the thermometer bulb. Insert the thermometer, together with the stirrer, and lower the test tube into the larger tube. A small quantity of alcohol, sufficient to fill the lower space between the two test tubes, will serve to complete the conduction medium between the freezing bath and the liquid to be tested. Keep the stirrer in steady up-and-down motion at a rate of approximately one stroke each 1 or 2 seconds, or even at a slower rate, providing the cooling proceeds satisfactorily. Maintain a passage of air through the apparatus until the temperature of the cooling-bath reaches -2.5° , at which time the top of the Hg thread in the thermometer usually recedes to a position near the freezing point of H_2O . Maintain the temperature of the cooling-bath at -2.5° , and continue the manipulation of the stirrer until a supercooling of sample of 1.0° to 1.2° is observed. As a rule, at this time the liquid will begin to freeze, as may be noted by the rapid rise of the Hg. Manipulate the stirrer slowly and carefully three or four times as the Hg column approaches its highest point. By means of a suitable light-weight cork mallet tap the upper end of the

thermometer cautiously a number of times until the top of the Hg column remains stationary for at least 1 minute. Observe the exact reading on the thermometer scale, taking necessary precautions to avoid parallax, and estimate to 0.001° . When the observation has been satisfactorily completed, make a duplicate determination; then remove the thermometer and stirrer and empty the H_2O from the freezing tube.

Laboratory Thermometer No. 2

Water	7 grams Sucrose to 100 ml.	10 grams Sucrose to 100 ml.
Av. $+0.056^\circ$	-0.425°	-0.621°

Interval = 0.196

0.196 equiv. 0.199

Correction factor = 1.015

Laboratory Thermometer No. 24

Water	7 grams Sucrose to 100 ml.	10 grams Sucrose to 100 ml.
Av. 0.000	-0.420°	-0.625°

Interval = 0.205

0.205 equiv. 0.199

Correction factor = 0.971

Example:

Laboratory Thermometer No. 24

F. pt. Depression Sample Milk = 0.548

 $(0.548 - 0.420) \cdot 0.971 = 0.124$ True F. pt. = $0.422 + 0.124 = 0.546^\circ$ below zero C.

B.S. TESTED THERMOMETER

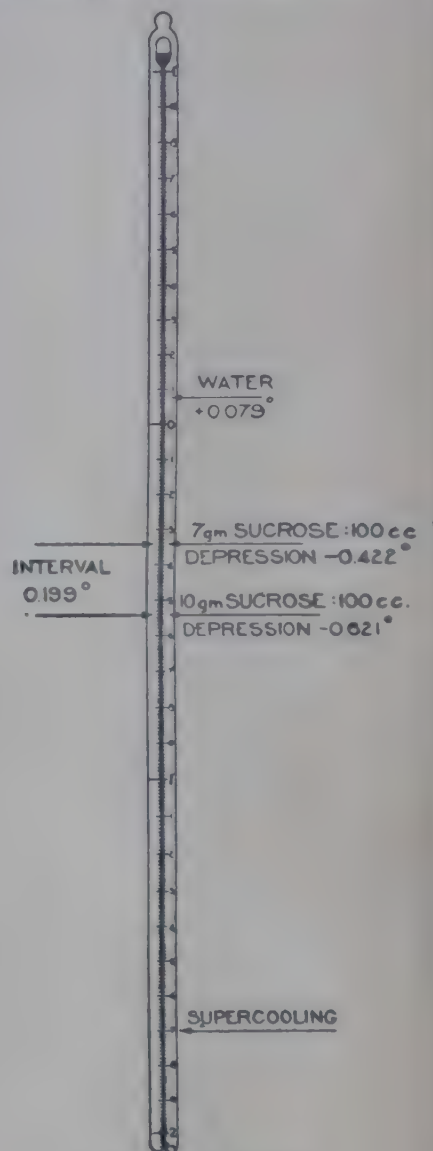


FIGURE XXXIII

Two Bureau of Standards tested thermometers gave intervals of 0.199° and 0.200° , respectively, between the freezing-point depression readings of the two sucrose solutions. One thermometer (Figure XXXIII) gave freezing-point depressions -0.422° ($+0.079$ and -0.343) and -0.621° ($+0.079$ and -0.542), respectively, for the two sucrose solutions, while the other gave -0.422° and -0.622° , respectively.

Rinse the tube with about 25 ml. of the sample of milk cooled to 10° or lower; measure into the tube 30–35 ml. of milk, or enough to submerge the thermometer bulb; and insert the tube into the apparatus. Maintain the temperature of the cooling-bath at 2.5° below the probable freezing point of the sample. Make the determination on the milk, following the same procedure as that employed in determining the freezing point of H_2O . As a rule, however, it is necessary to start the freezing action in the milk by inserting the freezing starter (which has been kept in contact with ice for several minutes, and in the open end of which has been wedged a fragment of ice) at the time when the Hg column has receded to 1.0 – 1.2° below the probable freezing point. A rapid rise of the Hg results almost immediately. Remove the starter and manipulate the stirrer slowly and carefully two or three times while the Hg approaches its highest point. Complete the adjustment of the Hg column in the same manner as in the preceding determination; then, avoiding parallax, observe the exact reading on the thermometer scale and estimate to 0.001° . The algebraic difference between the average of readings obtained on the H_2O and the reading obtained on the sample of milk represents the freezing-point depression of the milk. To determine the true freezing-point (T^1) of the milk, subtract from the freezing-point depression, the freezing-point depression of 7 per cent sucrose solution as determined by the laboratory thermometer. Multiply the difference by the correction factor for the thermometer. Add to the product 0.422 (freezing-point of 7 per cent sucrose solution by Bureau Standards thermometer). See example under Figure XXXIII.

Ascertain the percentage of added $H_2O(W)$ corresponding to the determined freezing-point depression from Table 22 under XLIII, pp. 717, 718, Methods of Analysis, A.O.A.C., 1940, or by means of the following formula:

$$W = \frac{100(T - T^1)}{T}, \text{ in which}$$

T = the average freezing point of normal milk (-0.550°), and
 T^1 = the true freezing point on a given sample.

A tolerance of 3 per cent may be allowed on results for added H_2O determined on the basis of an average freezing-point depression of -0.550° . Owing to the narrow variations found among market milks of genuine character it is not necessary to deduct the tolerance figure from results showing added H_2O in excess of 3 per cent.

XIV. GELATIN

1. QUALITATIVE TEST—OFFICIAL

To 10 ml. of the milk add an equal volume of acid $\text{Hg}(\text{NO}_3)_2$ solution (Hg dissolved in twice its weight of HNO_3 and this solution diluted to 25 times its volume with H_2O), shake the mixture, add 20 ml. of H_2O , shake again, allow to stand 5 minutes, and filter. If much gelatin is present, the filtrate will be opalescent and cannot be obtained quite clear. To a portion of the filtrate contained in a test tube add an equal volume of saturated aqueous picric acid solution. A yellow precipitate will be produced in the presence of any considerable quantity of gelatin, while smaller quantities will be indicated by a cloudiness.

NOTE: In applying this test to sour, fermented, cultured, or very old samples of milk, cream, or buttermilk, or to sterilized cream or evaporated milk, use care to recognize precipitates produced by picric acid when added to the $\text{Hg}(\text{NO}_3)_2$ filtrates from these materials in the absence of gelatin. (Such samples, with or without rennet and entirely free from gelatin, give, on standing, distinct precipitates when treated as above outlined. In every case, however, these precipitates differ in character from that which picric acid produces with gelatin. The gelatin-picric acid precipitate is finely divided, more apt to remain in suspension, settles only slowly, and adheres tenaciously to the sides and bottom of the container, from which it is rinsed with difficulty. Precipitates produced by picric acid in the absence of gelatin are flocculent, separate readily, leaving the serum practically clear, do not adhere to the walls of the container, and are easily removed by rinsing with H_2O . When gelatin is present in the sample, the gelatin-picric acid precipitate will remain in suspension long after the flocculent precipitate has settled, but on standing overnight the characteristic sticky deposit will be found adhering tenaciously to the bottom and sides of the test vessel. If gelatin is present in relatively high concentration (1 per cent), the gelatin-picric acid precipitate will be voluminous and will settle rather quickly.)

XV. PRESERVATIVES

1. FORMALDEHYDE

A. PHENYLHYDRAZIN HYDROCHLORIDE TEST¹²—OFFICIAL

To a portion of the sample add an equal volume of strong alcohol, shake, and filter from any insoluble matter. To 5 ml. of the filtrate add 0.03 gm. of phenylhydrazin hydrochloride and 4 or 5 drops of a 1 per cent FeCl_3 solution. Mix, add slowly with agitation, in a bath of cold H_2O to prevent heating the liquid, 1–2 ml. of H_2SO_4 . Dissolve the precipitate by the addition of either H_2SO_4 (keeping

the mixture cool) or alcohol. In the presence of HCHO a red color develops.

This method gives reliable reactions for HCHO in solutions of HCHO varying from 1 part in 50,000 to 1 part in 150,000. Neither acetaldehyde nor benzaldehyde interferes with the reaction.

B. LEACH TEST—OFFICIAL

Mix in a porcelain casserole about 10 ml. of the milk with an equal volume of HCl containing 1 ml. of 10 per cent FeCl_3 solution to each 500 ml. of acid. Heat slowly to $80-90^\circ$ directly over a gas flame, rotating the casserole to break up the curd. A violet color indicates formaldehyde.

C. HEHNER TEST¹³—OFFICIAL

To about 10 ml. of the milk in a wide test tube, add about half the volume of commercial H_2SO_4 , pouring the acid carefully down the side of the tube so that it forms a layer at the bottom without mixing with the milk. A violet or blue color at the junction of the two liquids indicates HCHO . The test is given only in the presence of a trace of FeCl_3 or other oxidizing agent. This test may be combined with the Babcock test for fat, noting whether a violet color forms on addition of the commercial H_2SO_4 to the milk in the test bottle. The test is sensitive to one or more parts of HCHO per 10,000.

D. PHENYLHYDRAZIN HYDROCHLORIDE AND SODIUM NITROPRUSSIDE TEST¹⁴—OFFICIAL

Dissolve a lump of phenylhydrazin hydrochloride about the size of a pea in 3–5 ml. of the milk to be tested, add 2–4 drops (not more) of a 5–10 per cent Na-nitroprusside solution and 8–12 drops of an approximately 10 per cent NaOH solution. If HCHO is present, a green or blue color develops, depending upon the quantity. When HCHO is present to the extent of more than 1 part in 70,000–80,000 in the solution tested, a distinct green or bluish green coloration is obtained. In more dilute solutions the green tint becomes less marked and a yellow tinge tending toward greenish brown develops. With this test acetaldehyde and benzaldehyde give a color varying, according to the strength of the solution, from red to brown. Therefore a reaction may be obtained with these aldehydes

similar to that obtained with HCHO in solutions more dilute than 1 part in 70,000. The presence of acetaldehyde or benzaldehyde together with HCHO gives a yellowish or yellowish green tinge. The reaction for HCHO , therefore, may be masked by the presence of other aldehydes, but it is characteristic when a clear green color is obtained.

E. PHENYLHYDRAZIN HYDROCHLORIDE AND POTASSIUM FERRICYANIDE
TEST ¹⁴—OFFICIAL

Proceed as directed under XV, D, substituting a solution of K-ferricyanide for the Na-nitroprusside. Formaldehyde gives a red color.

F. PHENYLHYDRAZIN HYDROCHLORIDE AND FERRIC CHLORIDE TEST ¹⁴
—OFFICIAL

Treat 15 ml. of milk with 1 ml. of a 1 per cent phenylhydrazin hydrochloride solution, then with a few drops of 1 per cent FeCl_3 solution and finally with HCl . The presence of HCHO is indicated by the formation of a red color, which changes after some time to orange yellow.

Milk may be examined directly by this method, but more delicate tests may be obtained from the distillate from milk or from milk serum. Acetaldehyde or benzaldehyde does not interfere with the reaction.

G. PHLOROGLUCOL TEST ¹⁵—OFFICIAL

To 10 ml. of milk in a test tube add, by means of a pipette, 2 ml. of phloroglucol reagent (1 gm. of phloroglucol, 20 gm. of NaOH , and H_2O to make 100 ml.), placing the end of the pipette on the bottom of the tube in such a manner that the reagent will form a separate layer. If HCHO is present, a bright red coloration (not purple) forms at the zone of contact. The clear, red color given by the use of this reagent forms quickly and, in the presence of but a small quantity of HCHO , fades rapidly.

2. SALICYLIC ACID

A. FERRIC CHLORIDE TEST—OFFICIAL

Acidify 100 ml. of the milk with 5 ml. of HCl (1 + 3), shake until curdled, filter, and extract with 50–100 ml. of ether. Wash the ether layer with two 5 ml. portions of H_2O , evaporate the greater

portion of the ether in a porcelain dish on a steam bath, allow the remainder to evaporate spontaneously, and add a drop of 0.5 per cent neutral FeCl_3 solution. A violet color indicates salicylic acid.

B. JORISSEN TEST¹⁶—OFFICIAL

Acidify, filter, and extract a portion of the milk as directed under XV, 2, A. Dissolve the residue from the ether extract in a little hot H_2O . Cool 10 ml. of the solution in a test tube, add 4 or 5 drops of a 10 per cent KNO_2 solution, 4 or 5 drops of 50 per cent acetic acid, and 1 drop of a 1 per cent CuSO_4 solution; mix thoroughly, and heat to boiling. Boil for $\frac{1}{2}$ minute and allow to stand for 2 minutes. In the presence of salicylic acid a Bordeaux red color develops.

3. BENZOIC ACID—OFFICIAL

Acidify, filter, and extract a 100 ml. portion of the milk with ether as directed under XV, 2, A. If benzoic acid is present in considerable quantity, it will crystallize from the ether in shining leaflets having a characteristic odor on warming. Dissolve the crystalline deposit in hot H_2O , divide into two portions, and test as directed below. The deposit may also be purified by sublimation and the melting point determined.

a. Make the solution alkaline with a few drops of NH_4OH , expel the excess of ammonia by evaporation, dissolve the residue in a few ml. of hot H_2O , filter, if necessary, and add a few drops of a neutral 0.5 per cent FeCl_3 solution. A brownish precipitate of ferric benzoate indicates the presence of benzoic acid.

b.¹⁷ Add to the H_2O solution 1 or 2 drops of a 10 per cent NaOH solution and evaporate to dryness. To the residue add 5–10 drops of H_2SO_4 and a small crystal of KNO_3 . Heat for 10 minutes in a glycerol bath at $120\text{--}130^\circ$. The temperature must not exceed 130° . After cooling, add 1 ml. of H_2O and make distinctly ammoniacal; boil the solution to decompose any NH_4NO_2 that may have been formed. Cool and add a drop of fresh colorless $(\text{NH}_4)_2\text{S}$, without allowing the layers to mix. A red-brown ring indicates benzoic acid. On mixing, the color diffuses through the whole liquid and, on heating, finally changes to greenish yellow. This differentiates benzoic acid from salicylic acid or cinnamic acid. The last two form colored compounds, which are not destroyed by heating.

4. BORIC ACID

A. PRELIMINARY TEST—OFFICIAL

Immerse a strip of turmeric paper in the sample acidified with HCl in the proportion of 7 ml. of strong acid to each 100 ml. of sample, and allow the paper to dry spontaneously. If borax or boric acid is present, the paper will acquire a characteristic red color, changed by NH_4OH to a dark blue-green, but restored by acid.

B. CONFIRMATORY TEST—OFFICIAL

Make about 25 ml. of the sample decidedly alkaline with lime water and evaporate to dryness on a steam bath. Ignite the residue at a low red heat until the organic matter is thoroughly charred. Cool, digest with about 15 ml. of H_2O and add HCl dropwise until the solution is distinctly acid. Saturate a piece of turmeric paper with the solution, and allow it to dry without the aid of heat. In the presence of borax or boric acid, the color change will be the same as described under the preliminary test.

XVI. COLORING MATTERS ¹⁸1. ANNATTO—OFFICIAL ¹⁹

Warm about 150 ml. of milk in a casserole over a flame and add about 5 ml. of acetic acid ($1 + 3$), then slowly continue the heating nearly to the boiling point while stirring. Gather the curd, when possible, into one mass with a stirring rod, and pour off the whey. If the curd breaks up into small flecks, separate from the whey by straining through a sieve or colander. Press the curd free from adhering liquid, transfer to a small flask, macerate, allow to stand for several hours (preferably overnight) in about 50 ml. of ether, keeping the flask tightly corked and shaking at intervals. Decant the ether extract into an evaporating dish, remove the ether by evaporation, make the residue alkaline with NaOH, and pour upon a small wet filter. If annatto is present, the filter paper will absorb the color so that, when washed with a gentle stream of H_2O , it will remain dyed a straw color. Dry the filter and add a drop of SnCl_2 solution. If the color turns pink, the presence of annatto is confirmed.

2. COAL TAR DYES—OFFICIAL

The curd of an uncolored milk is perfectly white after complete extraction with ether, as is also that of a milk colored with annatto. If the extracted fat-free curd is distinctly orange or yellowish in color, a coal tar dye is indicated. In many cases upon treating a lump of a fat-free curd in a test tube with a little HCl the color changes to pink, indicating the presence of a dye similar to aniline yellow or butter yellow or perhaps one of the acid azo yellows or oranges. If aniline yellow, butter yellow, or any other oil-soluble dye is present, the greater part will be found in the ether extract containing the fat.

In some cases the presence of coal tar dyes can be detected by treating about 100 ml. of the milk directly with an equal volume of HCl in a porcelain casserole, giving the dish a slight rotary motion. In the presence of some dyes the separated curd acquires a pink coloration.

For methods for the separation and identification of coal tar dyes used in foods, see *Methods of Analysis*, A.O.A.C., 1940, pp. 239–268.

XVII. SEDIMENT TEST ²⁰

See Part I, p. 68.

(This method was adopted by the Association of Official Agricultural Chemists and edited to conform in part to the style of "Methods of Analysis" of that Association.)

B. CREAM

XVIII. COLLECTION OF SAMPLE — OFFICIAL

Proceed as directed under I. Analyze the sample as soon as practicable, preferably not later than 3 days after taking.

XIX. PREPARATION OF SAMPLE — OFFICIAL

Immediately before withdrawing portions for the determinations, mix the sample by shaking, pouring, or stirring until it pours readily and a uniform emulsion has been secured. If the sample is very thick, warm it to 30–35°, and then mix. In case lumps of butter have separated, heat the sample to 38° or, if necessary, to 50° by placing in a warm water bath. Thoroughly mix the portions for analysis and weigh immediately. (In commercial testing for fat by the Babcock method, it may be advisable to warm all samples to 38–50° in a water bath previous to mixing.) Avoid overheating the sample, thereby causing the cream to “oil off.” This precaution is especially necessary in the case of a thin cream.

XX. TOTAL SOLIDS — OFFICIAL

Proceed as directed under VI, using 2–3 gm. of the sample.

XXI. ADDED WATER IN CREAM²¹ — OFFICIAL

Proceed as directed under XIII, 4, but use the following formula to calculate the percentage of added H₂O:

$$W = \frac{\% \text{ Serum in Cream } (T - T^1)}{T}, \text{ in which}$$

W = the percentage of added H₂O;

T = the freezing point of undiluted cream (–0.550°);

T¹ = the observed freezing-point of the given sample; and % serum = 100% – (% fat + % protein).

If protein is not determined it may be assumed to be 38 per cent of the solids-not-fat.

XXII. ASH — OFFICIAL

Proceed as directed under VII.

XXIII. TOTAL NITROGEN — OFFICIAL

Proceed as directed under VIII.

XXIV. LACTOSE**GRAVIMETRIC METHOD—OFFICIAL**

Proceed as directed under XI, 2.

XXV. FAT**1. ROESE-GOTTLIEB METHOD—OFFICIAL**

Transfer 5 gm. of the sample to a Röhrig tube or a similar apparatus, dilute with H_2O to about 10.5 ml. and proceed as directed under XII.

2. BABCOCK METHOD⁶—OFFICIAL**A. REAGENTS**

- (a) Sulfuric acid—Sp. gr. 1.82–1.83 at 20°.
- (b) Glymol, or clear white mineral oil—Sp. gr. not to exceed 0.85 at 20°. Oil-soluble artificial color may be added to the oil.

B. APPARATUS

a. *Test bottles*—The standard Babcock test bottles for cream shall be as follows:

(1) Fifty per cent, 9 gm., short-necked, 6 in. cream-test bottle—Total height 150–165 mm. (5.9–6.5 in.). The bottom of the bottle shall be flat, and the axis of the neck shall be vertical when the bottle stands on a level surface. The charge of cream for the bottle shall be 9 gm.

Bulb—The capacity of the bulb to the junction with the neck shall be not less than 45 ml. The shape of the bulb shall be either cylindrical or conical. If cylindrical, the outside diameter shall be between 34 and 36 mm.; if conical, the outside diameter of the base shall be between 31 and 33 mm., and the maximum diameter between 35 and 37 mm.

Neck—The neck shall be cylindrical and of uniform diameter from at least 5 mm. below the lowest graduation mark to at least 5 mm. above the highest. The top of the neck shall be flared to a diameter of not less than 15 mm. The graduated portion of the neck shall have a length of not less than 63.5 mm. The total per cent graduation shall be 50. The graduations shall represent 5 per cent, 1 per cent, and $\frac{1}{2}$ per cent, respectively, from 0.0 to 50 per cent. The 5 per cent graduations shall extend at least half-way around

the neck to the right; the $\frac{1}{2}$ per cent graduations shall be not less than 3 mm. in length; and the 1 per cent graduations shall be intermediate in length between the 5 per cent and $\frac{1}{2}$ per cent graduations and shall project 2 mm. to the left of the $\frac{1}{2}$ per cent graduations. Each 5 per cent graduation shall be numbered (thus: 0, 5, 10, 45, 50), the number being placed to the left of the scale. The capacity of the neck for each whole per cent on the scale shall be 0.1 ml. The maximum error in the total graduation or any part thereof shall not exceed the volume of the smallest unit of the graduation.

(2) Fifty per cent, 9 gm., long-necked, 9 in. cream-test bottle—The same specifications shall apply to this bottle as to the 50 per cent, 9 gm., 6 in. cream-test bottle, except that the total height of this bottle shall be 210–229 mm. (8.25–9.0 in.) and the graduated portion of the neck shall have a length of not less than 120 mm.

(3) Fifty per cent, 18 gm., long-necked, 9 in. cream-test bottle—The same specifications shall apply to this bottle as to the 50 per cent, 9 gm., 9 in. cream-test bottle, except that the charge of cream for this bottle shall be 18 gm.

Each bottle shall bear on the top of the neck above the graduations, in plain legible characters, a mark denoting the weight of the charge to be used, viz., “9 gm.” or “18 gm.,” as the case may be.

Each bottle shall be so constructed as to withstand the stress to which it will be subjected in the centrifuge.

(4) Testing—Proceed as directed under XII, 2, B, a, (1).

b. *Water bath for cream samples*—Provided with a thermometer and a device for maintaining a temperature of 38–50°.

c. *Cream weighing scales*—With a sensibility reciprocal of 30 mg., i.e. the addition of 30 mg. to either pan of the scale, when loaded to capacity, shall cause a deflection of at least 1 subdivision of the graduation. The scales shall be set level upon a table support and be protected from drafts.

d. *Weights*—9 gm. and 18 gm., respectively, and plainly marked “9 gm.” or “18 gm.,” as the case may be. They shall be made of material capable of resisting corrosion or other injury, shall preferably be of a low squat shape, with rounded edges, and shall be verified at frequent intervals by comparison with standardized weights.

- e. *Acid measure*—Described under XII, 2, B, c.
- f. *Centrifuge or "tester"*—Described under XII, 2, B, d.
- g. *Dividers or calipers*—Described under XII, 2, B, e.
- h. *Water bath for test bottles*—Described under XII, 2, B, f.

C. DETERMINATION

Weigh 9 gm. of the sample, prepared as directed under XIX, directly into a 9 gm. cream-test bottle, or 18 gm. into an 18 gm. bottle, and proceed by one of the following methods:

Method 1—After the cream has been weighed into the test bottle, add 8–12 ml. of the H_2SO_4 , in the case of the 9 gm. bottle, or 14–17 ml. of the acid, in the case of the 18 gm. bottle, or add acid until the mixture of cream and acid, after shaking, has assumed a chocolate-brown color. Shake until all lumps have completely disappeared; then add 5–10 ml. of soft H_2O at 60° or above. Transfer the bottle to the centrifuge; counterbalance it; and, after the proper speed has been attained, whirl 5 minutes. Add hot H_2O until the liquid column approaches the top graduation of the scale; then whirl 1 minute longer at a temperature of $55\text{--}60^\circ$. Adjust the temperature as directed under XII, and, with the aid of dividers or calipers, measure the fat column, in terms of percentage by weight, from its lower surface to the bottom of the upper meniscus.

Method 2—For a 9 gm. bottle only—After the cream has been weighed into the test bottle, add 9 ml. of soft H_2O and thoroughly mix; add 17.5 ml. of the H_2SO_4 , and shake until all lumps have completely disappeared. Transfer the bottle to the centrifuge; counter-balance it; and, after the proper speed has been attained, whirl 5 minutes. Fill the bottle to the neck with hot H_2O and whirl 2 minutes. Add hot H_2O until the liquid column approaches the top graduation of the scale; then whirl 1 minute longer at a temperature of $55\text{--}60^\circ$. Adjust the temperature and measure the fat column as directed under Method I.

Whichever method is followed, the fat column at the time of reading should be translucent, of a golden yellow to amber color, and free from visible suspended particles. All tests in which the fat column is milky or shows the presence of curd or of charred matter, or in which the reading is indistinct or uncertain, should be rejected.

If glymol is used, a few drops only should be introduced into the bottle just before the reading is made; it must not be dropped in, but must be allowed to flow down the side of the neck. For the purpose of measurement, the surface separating the glymol and the fat is regarded as representing the upper limit of the column.

XXVI. GELATIN — OFFICIAL

Proceed as directed under XIV. Observe note.

XXVII. PRESERVATIVES — OFFICIAL

Proceed as directed under XV.

XXVIII. COLORING MATTERS — OFFICIAL

Proceed as directed under XVI.

C. ICE CREAM (PLAIN)

XXIX. PREPARATION OF SAMPLE — OFFICIAL

Allow the sample to soften at room temperature. Owing to the fact that melted butter fat tends to separate out and rise to the surface, it is not advisable to soften the ice cream by heating on a water bath or over a flame. Mix thoroughly by stirring with a spoon or egg beater or by pouring back and forth between beakers.

XXX. NITROGEN — OFFICIAL

Place 4–5 gm. of the sample in a digestion flask and proceed as directed under VIII.

XXXI. TOTAL SOLIDS—TENTATIVE

Proceed as directed under VI, 1, but without sand, using 1–2 gm. of sample. The sample may be transferred for weighing by means of a short, bent, 2 ml. measuring pipette. Consider as total solids the residue obtained after heating for 4 hours.

XXXII. FAT

1. ROESE-GOTTLIEB METHOD—OFFICIAL

Weigh 4 gm. of the thoroughly mixed sample into a small dry beaker; add 3 ml. of H_2O ; thoroughly mix with a glass rod; and transfer to a Röhrig tube or a similar apparatus, washing out the remaining portion with the aid of an additional 3 ml. of H_2O . Add 2 ml. of NH_4OH , mix thoroughly and heat in a water bath at 60° . From this point proceed as directed under XII, 1, beginning with "Add 10 ml. of 95 per cent alcohol and mix well."

XXXIII. COLORING MATTERS — TENTATIVE

Curdle 150–200 gm. of the melted sample by adding an equal volume of H_2O and 10–20 ml. of acetic acid. Heat the mixture to 70 – 80° , stirring meanwhile, and allow to cool. Continue as directed under XVI.

D. MISCELLANEOUS

(Methods taken from *Wiley's Principles and Practice of Agricultural Analysis* are cited with permission of the Board of Editors of the Association of Official Agricultural Chemists. The method taken from the *U. S. Pharmacopoeia* is cited with permission of the Revision Committee of the *Pharmacopoeia*.)

XXXIV. AVAILABLE CHLORINE

(In preparations used for sterilizing milk equipment and utensils.)

A. REAGENTS

a. *0.1 N Na-arsenite solution* ²²—Use pure resublimed As_2O_3 , which has been dried to constant weight at 105° . Dissolve exactly 4.948 gm. of this pure As_2O_3 in 250–300 ml. of distilled H_2O in which has been dissolved 10 gm. of anhydrous Na_2CO_3 . In order to hasten the solution, heat the mixture and stir thoroughly, but avoid extended boiling. When all the As_2O_3 is dissolved, indicated by a clear solution, cool and make to volume in a liter volumetric flask. The solution should need no further standardization, but if desired, check against standard I solution, using starch solution as an indicator.

b. *0.1 N thiosulfate solution* ²³—Prepare by dissolving 26 gm. of crystallized $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ in recently boiled and cooled H_2O , filtering, and diluting to 1 liter with recently boiled and cooled H_2O . Standardize against a standard I solution, using starch solution as indicator.

c. *Starch-iodide paper* ²⁴—Saturate strips of filter paper in a starch-iodide solution prepared as follows: Mix about 1 gm. of finely powdered potato starch with cold H_2O to a thin paste, pour into about 100 ml. of boiling H_2O with constant stirring, immediately discontinue heating, and add 10 ml. of a 1 per cent solution of KI. (The paper should be damp during use.)

d. *Potassium iodide solution* ²⁵—Dissolve 16.5 gm. of KI in H_2O and dilute to 100 ml.

1. SODIUM HYPOCHLORITE SOLUTION ²⁶

This solution occurs in commerce in various strengths. The method described here is based upon a Na-hypochlorite content of approximately 5 per cent. For samples containing a different proportion of Na-hypochlorite the quantity taken for analysis should be varied accordingly.

A. DETERMINATION

Transfer a 20 ml. aliquot of the sample to a liter volumetric flask and dilute to volume. Pipette a 50 ml. aliquot of the diluted solution into a 200 ml. Erlenmeyer flask, and titrate with 0.1 N Na-arsenite solution, using starch iodide paper as an outside indicator. From the number of ml. of Na-arsenite solution used and the specific gravity of the hypochlorite solution (determined at 20°/4° by means of a Westphal balance, pycnometer or other convenient apparatus), calculate the percentage of Na-hypochlorite in the sample on the basis that 1 ml. of 0.1 N Na-arsenite = 0.003723 gm. of Na-hypochlorite.

Calculate available Cl from the Na-arsenite titration on the basis that 1 ml. = 0.003546 gm. of available Cl.

2. CHLORAMINE T ²⁷

A. DETERMINATION

Thoroughly mix the sample and dissolve about 0.5 gm., accurately weighed, in 50 ml. of H₂O, add 5 ml. of the KI solution and 5 ml. of acetic acid, and allow the mixture to stand in a glass-stoppered bottle for 10 minutes. Titrate the liberated I with 0.1 N Na-thio-sulfate, using starch solution as indicator. Each ml. of 0.1 N Na-thiosulfate corresponds to 0.001773 gm. of active Cl. From this titration calculate the percentage of active Cl in the sample.

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APPENDIX

*Phosphatase Methods
not yet studied
or approved
as Standard
Methods by the
American Public
Health Association*

APPENDIX

PROPOSED PHOSPHATASE METHODS FOR DETERMINATION OF PASTEURIZATION

(American Public Health Association)

INTRODUCTION

For the convenience of laboratory workers, directions for making phosphatase tests to determine the efficiency of pasteurization have been included in this appendix. This test is based on the property of the heat-sensitive enzyme phosphatase to liberate phenol from phosphoric-phenyl esters. When milk is heated, this enzyme becomes progressively inactivated; when heated at 143° F. for 30 minutes, 96 per cent of the enzyme is destroyed, and heating above 145° F. for 30 minutes insures complete inactivation. When the milk has been under-heated (in temperature and/or time) or when there is an admixture of raw milk, the enzyme will be present in larger amounts than when the milk was properly processed and handled. The amount of phosphatase present is measured colorimetrically, and the quantitative determination reveals the degree of faulty pasteurization or subsequent contamination with raw milk. Over all ranges of temperature and times, *Mycobacterium tuberculosis* is destroyed more quickly than phosphatase so that a heat treatment adequate to inactivate the enzyme to the degree called for by the standard likewise kills this organism and all other common pathogenic bacteria. A sample of milk that does not have more phosphatase present than the standard allows can be regarded as safely pasteurized and free from subsequent contamination with raw milk.

Since the first publication of this test, several modifications of the technic have been devised. Each has contributed its part to further development of our knowledge of the field. All of them are being used by regulatory and industrial quality control officials, but information as to their relative accuracy, dependability, and utility is only beginning to make its appearance.

The first of these technics was proposed by Kay and Graham¹ of the Dairy Research Institute at Shinfield near Reading, England. The first methods suggested were soon revised and improved,^{2, 3} a short method being proposed that was sufficiently accurate to deter-

mine within a few minutes whether a milk has or has not been heated, and a second longer or laboratory test (18 to 24 hours). The latter was reported to be sufficiently accurate to detect under-heating of $1\frac{1}{2}$ degrees, shortening of time from 30 to 20 minutes, or the addition of 0.25 per cent raw milk. Results are read by using a Lovibond Tintometer with colored glass discs. Recently (1939) Kay and Neave⁴ have simplified this technic still further. By using a 4.5 per cent solution of sodium hexametaphosphate to dilute the Folin-Ciocalteu reagent the filtration subsequent to the addition of the sodium carbonate is obviated, and the procedure shortened by one filtration. Through the courtesy of these authors this modification was published in the Seventh Edition of this Report for the first time.

The original longer or laboratory form of this procedure was studied by Gilcreas and Davis⁵ of the Division of Laboratories and Research of the New York State Department of Health who developed liquid color standards. The Kay-Graham test as modified by them has been made official for New York State outside of New York City.

Meanwhile, Scharer⁶ and his associates in the City of New York Dept. of Health, utilizing a differently buffered substrate and the especially sensitive indicator used by water analysts, developed a so-called field test (results secured in 10-30 minutes) and a laboratory test (results secured in one hour) for the determination of phosphatase activity. The official procedure in the City of New York has been to test collected samples by the laboratory procedure first. Where inadequate pasteurization or admixture of raw milk is indicated, inspectors return to secure additional samples and test them immediately with the field test. If results secured with the field test indicate that faulty conditions still exist, an embargo is placed on the milk.

More recently, Leahy⁷ of the Rochester, N. Y., Health Bureau Laboratories, has modified the tests previously suggested using the substrate suggested by Kay and Graham, and the indicator suggested by Scharer and his associates.

There is reason to believe that these procedures yield results of value to milk control officials in that they provide a method whereby the efficiency of pasteurization can be checked by an examination of the milk itself.⁸ There is reason to believe also that the results secured are reasonably accurate. "Short tests," and "field tests"

have been developed by the originators of these methods to increase their adaptability to inspection work.

Developments in this field have been rapid, and many individual municipal, state, and provincial departments of health have made comparative tests. Comprehensive reviews of the various phosphatase tests have been published by Burgwald,⁹ and Kay, Aschaffenburg, and Neave.¹⁰ Gilcreas^{11, 12} has made comparative studies of various forms of these tests for the Association of Official Agricultural Chemists. His studies have resulted in the recognition by that Association of the technic used by the New York State Department of Health (See Appendix, Method No. II, p. 266)¹¹ and the Rapid or Laboratory Phosphatase Test used by the New York City Department of Health (See Appendix, Method No. III, 1, Rapid Phosphatase Test, p. 270)¹² as Tentative Methods.

Burgwald's review,¹³ and Fasken and McClure's paper¹⁴ give an indication of the rapidity with which phosphatase tests have been introduced into municipal milk control programs in the United States and Canada. Out of 62 cities replying to a questionnaire sent to 100 of the larger cities, only 6 replied that they were not using some form of the phosphatase test in their routine work. Twenty-five were found to be making regular use of the New York City field test; 8 were making regular use of the New York City laboratory test, and 8 additional cities were using the laboratory test to check the field test. Eight cities were using the original Kay-Graham technic, 8 the Gilcreas and Davis modification, and 1 the Neave modification of the Kay-Graham technic. Some of the cities reported using the results of one technic as a check on the results secured by the other.

While the phosphatase test has been applied to cream, ice cream and other frozen desserts, butter, and cheese, results secured must be interpreted with caution because of the possibility that ^{13, 15} interfering substances, detectable by running controls may be present. It has been found that the growth of certain types of bacteria produce changes that interfere with results. These interfering substances normally cause false positive tests.

A. CAUTIONS TO BE OBSERVED

Inaccurate results from these tests arise because of carelessness of various types. The indicator used in the New York City and

Rochester technics is even more sensitive to traces of phenol than the indicator used in the Kay-Graham and New York State technics. Precautions to prevent contamination with phenol from extraneous sources must therefore be constantly observed.

a. Test the purity of reagents. The reagents now available from the sources indicated are much more satisfactory than were available commercially when these tests were first announced. The suitability and purity of so-called distilled waters should be tested.

b. For best results, the n-butyl alcohol used for extraction of the indophenol in the New York City field test method should be colorless, of good quality, and free of excessive acid or esters. If the alcohol utilized is very old, it should be redistilled with sodium hydroxide. To test the suitability of the alcohol, shake a sample with an equal quantity of neutral distilled water, allow it to separate, and test the water layer with brom thymol blue. A blue or green color should result. Errors are introduced where the n-butyl alcohol does not meet the standard specified or where the extraction process is not carried out carefully. Either too much or too little color may be obtained causing reports to show errors of both a too severe and too lenient a character.

c. Avoid contamination with phenol and phenolic compounds. Phenolic soaps, some types of plastic closures for bottles, some rubber or even cork stoppers may liberate enough phenol to be absorbed. Pure gum rubber stoppers are reported to be free from phenolic compounds.

d. When not in use, keep solutions well stoppered in a dark, cool place, and protect pouring lips from dust.*

e. Pipettes and other glassware must be clean and free from milk, particularly raw milk. Do not use pipettes a second time for milk without thorough cleaning.

f. Special filter paper is required for the Kay-Graham and New York State tests.

g. Keep samples well refrigerated to prevent growth of microorganisms which may adversely affect dependability of results. Examine sample preferably within 18 hours of collection.

h. Avoid chemical preservatives, except that borax may be used in samples examined by the New York City procedures.

* If saturated sodium carbonate solutions are stored at too low a temperature they will recrystallize.

i. Carefully observe all other directions given. Use controls wherever these are indicated.

METHOD NO. I

Modified Kay-Graham-Neave Technic^{3, 4}

The standards under this technic are based on a minimum pasteurization temperature of 145° F. for 30 minutes.*

A. COLLECTION OF SAMPLE

The quantity of sample required depends upon the number of determinations to be made. For the usual analysis collect 5–10 ml. or in the case of bottled milk, collect one or more bottles as prepared for sale. In sampling bulk milk, thoroughly mix by pouring from one clean vessel into another three or four times. If this procedure is impracticable, thoroughly stir the milk for at least 30 seconds with a suitable appliance long enough to reach to the bottom of the container. If cream has formed on the milk, continue the mixing until all cream is detached from the sides of the vessel and evenly emulsified throughout the liquid.

Place the samples in non-absorbent, airtight containers, and keep them in the cold, but at a temperature above freezing, until ready for examination. When transported by mail, express, or otherwise, completely fill the containers, tightly stopper, and mark for identification. Do not use preservatives. Examine within 48 hrs.

B. REAGENTS

a. *Buffer substrate tablets* †—Dissolve 1 tablet in 50 ml. of distilled water saturated with pure chloroform. If not made up fresh on each occasion, keep solution in refrigerator with several extra drops of chloroform. Stable for several months.

b. *Folin-Ciocalteu phenol reagent* †—Dilute with twice its volume of a 4.5 per cent solution of sodium hexametaphosphate (prepared by dissolving the pure salt in warm distilled water, cooling, and making up to 100 ml. for every 4.5 gm. of salt present). The mixture remains serviceable for several days if kept well protected.⁴

c. *Sodium carbonate solution*—Dissolve 140 gm. (1.32 molar

* Some work in the United States has shown that the procedure outlined is adaptable for all ranges of pasteurization temperature and holding time by varying the incubation period.

† Obtainable from The British Drug Houses Limited. Also see directions for preparing these reagents in Method II, B, a and b.

solution) of pure anhydrous sodium carbonate in distilled water and make up to 1 liter.

C. APPARATUS

Test tubes.

Pipette, 1 ml.

Simple comparator,* with one or more standard colored Lovibond glasses, including 2.3 blue units. (Use of Lovibond tintometer with set of colored glasses and a 13 mm. cell will enable analyst to correlate readings with those of Kay and Graham.)

Filter papers, Whatman, 9 cm. No. 30 or No. 42 (preferably the latter).†

Water bath, kept at $47^{\circ}\text{C.} \pm 2^{\circ}\text{C.}$

Water bath or incubator, maintained at 37° to 38°C.

Pipettes, 0.5 ml. grade A or N.P.L. (A separate pipette is required for each milk sample.)

Test tubes, $\frac{5}{8}$ inch diameter, marked at 10 ml., with rubber stoppers to fit.

Filter funnels to take 9 cm. filter papers.

1. TEST A (Short Test)

This test can be carried out in $\frac{1}{2}$ hour, and is useful for determining whether a milk has been heated or not, and for observing gross errors of pasteurization technic.

A. DETERMINATION

To 10 ml. of the buffer substrate solution contained in a 25 ml. test tube, add 0.5 ml. of the well mixed milk, mix thoroughly, and incubate the mixture in a water bath at $47^{\circ} \pm 2^{\circ}\text{C.}$ for 10 minutes. Remove the tube and cool to 15° to 20°C. by immersion in cold water. Add 4.5 ml. of the diluted Folin-Ciocalteu reagent, allow to stand for 3 minutes and filter. To 10 ml. of the filtrate add 2 ml. of the sodium carbonate solution, mix and heat in boiling water (kept boiling) for 2 minutes.

The test should be carried out in duplicate, and at the same time duplicated control tests should be made in the following manner:

* Made by Tintometer, Limited, Salisbury, England.

† Preliminary tests indicate that Eaton-Dikeman Company acid-washed filter papers known as New Filt No. 1 or No. 3 are satisfactory substitutes. These are made at Mt. Holley Springs, Pa., and are sold by the usual laboratory supply houses.

B. CONTROL TESTS

Mix thoroughly 10 ml. of the buffer substrate solution with 4.5 ml. of the diluted Folin-Ciocalteu reagent and 0.5 ml. of the milk, allow to stand for 3 minutes, and filter. To 10 ml. of the filtrate add 2 ml. of the sodium carbonate solution, mix and heat in boiling water (kept boiling) for 2 minutes. Compare the colors of the four tubes with those of the standard glasses.

C. INTERPRETATION

If the controls show more than a trace of blue color and the reagents contain no free phenol, it is probable that a phenol-producing organism is present in the milk. This does *not* occur in pasteurized milk which has been kept at a satisfactorily low temperature following pasteurization. With fresh, properly pasteurized milk, that is, milk which has been cooled to 55° F. immediately after pasteurization and maintained between that temperature and 65° F. for not more than 18 hours, the control tubes should show only a trace of blue color. If with such controls the *incubated* tubes show a definite blue color, *i.e.*, over 2.3 units, it can be said with certainty that the milk has not been adequately pasteurized, and if the color is over 6.0 blue units, the milk has very probably not been heated at all. With some experience, it will be possible to omit the control tubes for the majority of samples submitted to this test.

While the production of a color exceeding the standard may be taken as evidence of imperfect pasteurization or admixture of raw milk, the converse is not invariably true. For example, if the milk has been heated to 140° F. instead of at (or above) the statutory minimum temperature of 145° F., the color produced in test A will not exceed the standard. In cases where the color approaches very near to the standard tint, or where more accurate information is required regarding the efficiency of pasteurization, and generally, when time permits, the following more delicate Test B should be applied.

2. TEST B (Control Laboratory Test)

This test detects small as well as gross errors in processing, and is the one most generally used in the control laboratory.

A. DETERMINATION

To 10 ml. of the buffer substrate solution contained in a 25 ml.

stoppered test tube, add 0.5 ml. of the well mixed milk, and mix thoroughly. Add 3 drops of chloroform, stopper the tube, and incubate at 37° C. to 38° C. for 24 hours. At the end of this time, cool, and add 4.5 ml. of the diluted Folin-Ciocalteu reagent, mix, allow to stand for 3 minutes, and filter. To 10 ml. of the filtrate add 2 ml. of the sodium carbonate solution, mix and heat in boiling water (kept boiling) for 2 minutes. Compare the color in the tintometer or comparator.

B. CONTROL TESTS

Tests should always be made in duplicate, and control tests carried out when necessary. The controls in Test B either should be completed within a few minutes as in Test A, or should be filtered at the stage reached after adding the Folin-Ciocalteu reagent and be kept in the refrigerator until the next day. In practice, a useful alternative is to keep the milk samples in the refrigerator for 24 hours after the two experimental tubes have been put into the incubator and only carry out controls the following day, on those samples which show a positive test after 24 hours incubation.

C. INTERPRETATION

Milk which, with controls below 1.5 units, give colors below 2.3 blue units may be classified as properly pasteurized. Those giving colors between 2.3 and 6.0 blue units should be classified as improperly pasteurized, while those giving colors greater than 6.0 show evidence of serious errors in pasteurization performance. Raw milk usually gives more than 30 units of color.

It is not possible by the phosphatase test alone to decide what is the specific cause of a high figure. All that can be said is that the milk has not been correctly pasteurized. The limits of accuracy claimed for Test B are as follows:

- a. Holding at 145° F. for 20 instead of 30 minutes.
- b. Holding at 1½° F. below the minimum temperature (*i.e.*, holding at 143.5° F. or below).
- c. Admixture of 0.2 per cent of raw milk with correctly pasteurized milk.

D. PRECAUTIONS

Where possible, the test should be carried out on samples of milk within 18 hours of pasteurization, although milk samples may be

stored in cold storage for several days to as long as 2 weeks without affecting the results.

Carbolic soaps, or phenol or disinfectant of a similar type should be kept away from the vicinity in which the tests are being performed.

Glassware should be carefully cleaned with hot soda solution, and rinsed thoroughly before use. The use of disinfectant soap should be strictly avoided.

E. OTHER APPLICATIONS

The phosphatase test may be applied directly to homogenized milk.

In the examination of cream, the latter should be diluted with water until the butter fat content is decreased to 4 to 6 per cent. The sample can then be examined by the phosphatase technic the same as for milk.

METHOD NO. II

*Method Developed and Used by the Department of
Health, State of New York*

*Now Recognized as a Tentative Method by the
Association of Official Agricultural Chemists*¹¹

A. COLLECTION OF SAMPLE

Proceed as directed, Part IV, A, I, p. 224, except that no preservative shall be present and not more than 48 hours shall have elapsed between the time of sampling and receipt at the laboratory. If samples are refrigerated, observe precautions to prevent freezing.

B. REAGENTS

a. *Buffer substrate*—Dissolve 1.09 gm. of disodium phenyl phosphate and 11.54 gm. of sodium veronal (sodium diethyl barbiturate) in water saturated with CHCl_3 and dilute to 1 liter. Add 10 ml. of CHCl_3 per liter and store the reagent in a refrigerator.

b. *Folin-Ciocalteu phenol reagent*—Dissolve 100 gm. of sodium tungstate, $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$ (according to Folin), and 25 gm. of sodium molybdate, $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, in 700 ml. of water in a 1,500 ml. flask connected by a ground-glass joint to a reflux condenser. Add 50 ml. of 85 per cent phosphoric acid and 100 ml. of HCl , and reflux gently for 10 hours. Cool, add 150 gm. of lithium sulfate, 50 ml. of

water and 4–6 drops of liquid bromine. Boil without condenser for 15 minutes to remove excess bromine. Cool, transfer to a 1 liter flask, dilute to volume with water, and filter. (The finished reagent should have a golden yellow color; reject the reagent if it has a greenish tint.) Keep in a refrigerator and protected from dust. For use dilute 1 vol. of this stock reagent with 2 vols. of water.

c. *Sodium carbonate solution*—Prepare a 14 per cent or 1.32 M solution of anhydrous Na_2CO_3 .

d. *Chloroform*.

e. *Standard color solutions*.

C. APPARATUS

Test tubes, 20 mm. \times 160 mm.

Test tubes, 13 mm. inside diameter

Paper toweling

Water bath

Incubator or bath at 34°–37° C.

Funnels to take 11 cm. filter paper

Filter paper must be free from phenol and other interfering substances. (Whatman No. 40, and Eaton and Dikeman "New Filt" Nos. 1 and 3 have been found satisfactory.)

Daylight lamp

Turquoise blue, unglazed, opaque glass plate

D. PERMANENT PHENOL STANDARDS

a. *Color solution, grey*—Dissolve in distilled H_2O 31.9 gm. Co chloride, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$; 67.5 gm. Cu sulfate, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$; 75 gm. Ni sulfate, $\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$; add 32 ml. $\ast\text{HCl}$ and 45 ml. H_2SO_4 ; and dilute to 500 ml.

b. *Color solution, red*—Dissolve 476 gm. of Co chloride, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, in distilled H_2O , and filter. To filtrate add 100 ml. $\ast\text{HCl}$ and dilute to 1 liter.

c. *Color solution, blue*—Dissolve 300 gm. of Cu sulfate, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, in distilled H_2O , add 20 ml. of $\ast\text{H}_2\text{SO}_4$, and dilute to 1 liter. (Should crystals appear when solution is cooled to below 20° C. warm slightly before using to insure complete solubility.)

\ast Concentrated.

Prepare permanent color standards equivalent to phenol concentrations of from 0.01 to 0.15 mg. per 0.5 ml. of sample by com-

TABLE I
PREPARATION OF PERMANENT PHENOL STANDARDS

Phenol	Color Solution		
	Grey (a)	Red (b)	Blue (c)
mg./0.5	ml.	ml.	ml.
0.01	0.30	0.106	0.96
0.02	0.40	0.140	1.16
0.03	0.55	0.180	1.65
0.04	0.65	0.216	2.10
0.06	0.92	0.286	3.00
0.09	1.30	0.326	4.40
0.12	1.70	0.360	5.70
0.15	2.50	0.396	7.10

binning the quantities of color solutions **a**, **b**, and **c** indicated in the subjoined table, and diluting to 10 ml. with distilled H₂O in each case; *e.g.*, 0.3 ml. solution **a**, + 0.106 ml. solution **b**, + 0.96 ml. solution **c**, + H₂O to make a vol. of 10 ml. which is equivalent to a phenol concentration of 0.01 mg. in 0.5 ml. of sample.

These color standards are suitable for use only in natural daylight. If, however, a turquoise blue, unglazed, opaque glass plate is used to deflect the light from a daylight lamp through the tubes of standards and sample, accurate color comparisons can be made in the absence of daylight. Since the standards are prepared for use only at 13 mm. depth of color, tubes of different diameters cannot be used for accurate work.

E. DETERMINATION

Transfer 10 ml. of the buffer substrate solution into a test tube 20 mm. × 160 mm. and add 0.5 ml. of the milk to be tested. Add a

few drops of CHCl_3 , mix thoroughly by rotating the tube, and cover to protect contents from dust. (Do not use rubber or cork stoppers; paper toweling placed over the open end of the tubes is satisfactory.) Warm to 37° to 39° C. in a water bath and incubate at 34° to 37° C. for not less than 18 and not more than 24 hours. After incubation add 4.5 ml. of the diluted Folin-Ciocalteu reagent. Mix and allow to stand for 3 minutes. Filter and transfer 5 ml. of the filtrate to a test tube of 13 mm. diameter. Add 1 ml. of the 14 per cent anhydrous Na_2CO_3 solution and mix thoroughly by rotating the tube. Place the tube in a boiling water bath for 5 minutes and filter. Cool and estimate the color of the filtrate by comparison with the permanent color standards.

F. CONTROL TEST

(To check deterioration of reagents and the presence of interfering substances in the milk sample.)

To 10 ml. of the buffer substrate solution add 4.5 ml. of the diluted Folin-Ciocalteu reagent and 0.5 ml. of the milk sample. (Do not incubate.) Mix thoroughly, allow to stand for 3 minutes and filter. To 5 ml. of the filtrate add 1 ml. of the 14 per cent Na_2CO_3 solution, mix thoroughly by rotating the tube, heat in a boiling water bath for 5 minutes and filter. Cool and compare the color of the filtrate with the permanent color standards. If the phenol value obtained is greater than 0.02 mg., subtract the excess from the phenol value of the incubated sample to obtain the phenol value indicative of pasteurization treatment.

G. INTERPRETATION

A phenol value of 0.04 mg. phenol per 0.5 ml. of sample generally indicates milk heated to 143° F. for 30 minutes. A value greater than this indicates progressively inadequate heat treatment. In reporting results, give the mg. phenol per 0.5 ml. of sample as well as an interpretation as to whether the milk is pasteurized or underpasteurized.*

* Recent work indicates that a phenol value of <0.05 mg. phenol per 0.5 ml. of sample generally indicates milk heated to 143° F., for 30 minutes (Gilcreas, *A.J.P.H.*, 29: 158 [Feb.], 1939).

METHOD NO. III

*Methods Developed and Used by the Department of Health,
City of New York*1. THE RAPID (LABORATORY) PHOSPHATASE TEST ⁶

*Now Recognized as a Tentative Method by the Association
of Official Agricultural Chemists.*¹²

A. COLLECTION OF SAMPLES

In sampling bulk or bottle milk, thoroughly mix by pouring from one clean vessel into another three or four times. If this procedure is impractical, thoroughly stir the milk for at least 30 seconds with a suitable appliance long enough to reach to bottom of container. If cream has formed on the milk, continue the mixing until all cream is detached from the sides of the vessel and evenly emulsified throughout the liquid.

Place samples in non-absorbent, airtight containers, and keep them in the cold but at a temperature above freezing, until ready for examination. If necessary, borax may be added as a preservative (0.8 gm. per 100 ml. milk). If samples sour, neutralize to pH 6.6 with Na_2CO_3 before proceeding with analysis.

B. REAGENTS

a. *Borate buffer*—Dissolve 28.427 gm. of sodium borate ($\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$) in 900 ml. of water. Add 3.27 gm. NaOH (81.75 ml. Normal NaOH solution) and dilute to 1 liter.

b. *Gibbs phenol reagent*—Dissolve 40 mg. 2,6-dibromoquinone-chloroimide (referred to below as BQC) in 10 ml. methyl or 95 per cent ethyl alcohol. Keep reagent tightly stoppered and under refrigeration.

c. *Lead acetate solution*—Dissolve 50 gm. of lead acetate [$\text{Pb}(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot 3\text{H}_2\text{O}$] in 100 ml. water.

d. *Buffer substrate*—Dissolve 0.5 gm. crystalline disodium phenyl phosphate in 5 ml. of water in a small (10 × 100 mm.) test tube. Add 0.5 ml. borate buffer. Shake well and add 0.05 ml. BQC solution. (Or add two drops from a dropper delivering 50 drops per ml. of the BQC solution.) Shake well. Allow 5 minutes for color development. Extract indophenol by shaking with 2 ml. neutral

n-butyl alcohol. Allow to stand until alcohol has separated completely. Remove supernatant alcohol layer with a pipette or medicine dropper and discard. Dilute remainder with 100 ml. borate buffer and sufficient water to make 1 liter. This buffer substrate is phenol-free. Store under refrigeration. Because of possible decomposition, prepare quantities of this reagent sufficient for immediate needs only. The pH of this solution is approximately 9.6 (blue to thymolphthalein solution—0.04 per cent in 50 per cent ethyl alcohol). Avoid intimate contact of solution with rubber (a darkening indicates decomposition).

Tablets are available for convenient preparation of small quantities of the buffer substrate and BQC solutions.*

e. *Sodium pyrophosphate solution*—Dissolve 10 gm. $\text{Na}_4\text{P}_2\text{O}_7 \cdot 10\text{H}_2\text{O}$ in 100 ml. water.

C. APPARATUS

Pipettes, 1 ml.

Test tubes, pyrex, 15 mm. \times 125 mm.

Test tubes, 150 \times 15 mm.

Incubator or water bath set at 37° to 45° C.

Water bath

Funnel

Filter paper (any inexpensive qualitative paper)

Dropper

Double fluxed glass or sheet of Plastacele No. C-1605 HH, 0.015 in. thick

Standard color solutions

D. PERMANENT COLOR STANDARDS

1. Laboratory Test—Acid Series

a. *Color solution, red*—Cobalt chloride in 1 per cent HCl (59.59 gm. $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ per liter).

b. *Color solution, blue*—30 per cent copper sulfate in 1 per cent HCl (300 gm. $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ per liter).

c. *Color solution, yellow*—0.5 N (M/6) ferric chloride in 1 per cent HCl (45.05 gm. $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ per liter).

Combine quantities indicated in table following and dilute to 5 ml. with water.

* Obtainable from The Applied Research Institute, 15 West 34th Street, New York, N. Y.

COLOR SOLUTIONS

Units	Blue ml.	Red ml.	Yellow ml.
1	0.2	0.35	0.5
2	0.5	0.6	0.55
3.5	0.7	0.5	0.5
5	1.0	0.75	0.5
7.5	1.5	0.75	0.5
10	2.0	1.0	0.25

The following ammoniacal color standards supplement the acid series above by affording a more extensive range.

2. Laboratory Test—Ammoniacal Series

a. *Color solution, red*—Dissolve 1.8 gm. roseo (aquopentamine cobaltic chloride $[\text{Co}(\text{NH}_3)_5 \cdot \text{H}_2\text{O}]\text{Cl}_3$ per liter of 2.8 per cent ammonium hydroxide.

b. *Color solution, yellow*—Dissolve 0.84 gm. ammonium chromate $(\text{NH}_4)_2\text{CrO}_4$ per liter of 2.8 per cent ammonium hydroxide.

c. *Color solution, blue*—6.24 gm. copper sulfate $(\text{CuSO}_4 \cdot 5\text{H}_2\text{O})$ per liter of 2.8 per cent ammonium hydroxide.

Combine quantities indicated in table following and dilute to 5 ml with water.

COLOR SOLUTIONS

Units	Blue ml.	Yellow ml.	Red ml.
1	0.25	0.4	0.5
2	0.5	0.5	0.75
3.5	0.8	0.7	1.0
5	1.0	0.55	0.6
7.5	1.25	0.75	0.75
10	1.5	0.5	0.25
15	2.5	0.5	
20	2.75	0.5	
25	3.0	0.5	
50	3.75	0.5	
100	4.0	0.5	
500	4.5	0.5	

3. Field Test

a. *Color solution, red*—Same as acid series above.

b. *Color solution, yellow*—Same as acid series above.

c. *Color solution, blue*—0.5 N Copper sulfate in 1 per cent HCl (62.425 gm. $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ per liter).

Combine quantities indicated in table following, and dilute to 5 ml. with water.

Units	COLOR SOLUTIONS		
	Red ml.	Blue ml.	Yellow ml.
2	0.4	1.5	0.5
5	0.2	2.2	0.5

These color standards are suitable for use in natural or artificial light provided an opal double fluxed glass or sheet of Plastacele No. C-1605 HH, 0.015 inch thick is used as a light filter. Test tubes similar to those in which each test is conducted should be used for color standards.

E. DETERMINATION

Transfer 1 ml. of the milk to be tested to a pyrex test tube (15 mm. \times 125 mm.) and add 10 ml. of the buffer substrate. Mix thoroughly. Warm to about 40° C., and place in an incubator or water bath for 1 hour at 37° to 45° C. (41° preferred). After incubation, place tube in boiling water for 5 minutes. Cool in ice water. Add 0.1 ml. lead acetate solution. Shake immediately and well. Proteins will coagulate and separate sharply. In some instances it may be necessary to add an additional 0.05 ml. of lead acetate. Filter. To 5 ml. of filtrate in a test tube (6 \times 5½ inches), add 0.5 ml. borate buffer. Addition of a few drops of the pyrophosphate solution will clarify filtrate turbidity if any. Add 0.04 ml. BQC solution (2 drops from recommended dropper). Mix thoroughly by rotating tube. After 15 minutes, estimate color by comparison with permanent color standards.

F. CONTROL TESTS

To check presence of interfering substances in milk sample. To 9 ml. of water add 1 ml. of borate buffer and 1 ml. of milk sample. Mix well, place in boiling water for 5 minutes, and follow through as in DETERMINATION. Development of blue color indicates extent of indophenol color due to interfering substances in milk sample.

To check reagents. To 5 ml. of buffer substrate, add 0.04 ml. of BQC solution. Development of blue color in 15 minutes indicates

extent of substrate decomposition. Or, incubate 10 ml. of buffer substrate with 1 ml. of a boiled milk sample. Proceed as under DETERMINATION. Indophenol development indicates extent of substrate decomposition. Avoid intimate contact of BQC solution with rubber. A darkening of this solution indicates decomposition.

G. INTERPRETATION

Milk heated to 143° F. for 30 minutes will yield less than 2 units of color. Commercially pasteurized milk usually yields 0 to 1 unit because of added time exposure incurred by operating variations in preheating, filling and emptying of tanks, etc. A value of 2 units or greater in commercially pasteurized milk indicates inadequate heat treatment.

2. FIELD TEST

Solutions of buffer substrate and BQC whether prepared from reagents or tablets (see above) may be used in the field test.

A. REAGENTS

- a. *Neutral n-butyl alcohol*—Boiling range 115° to 118° C.*
- b. *BQC solution*—See III, 1, B, b, p. 270. If tablets are used, one tablet is equivalent to 20 mg. BQC and should be dissolved in 5 ml. of ethyl or methyl alcohol.
- c. *Buffer substrate solution*—See III, 1, B, d, p. 270. If tablets are used: Crush buffered substrate tablet in test tube, dissolve in 5 ml. of distilled water. Add 2 drops of BQC solution. Allow 5 minutes for color development, then extract the indophenol with 2 to 2.5 ml. of normal butyl alcohol. Allow to stand until alcohol layer has separated at top of tube. Remove alcohol layer with medicine dropper and discard. Dilute remainder of solution to 50 ml. This solution is then phenol free.

B. APPARATUS

Test tubes 10 × 110 mm. calibrated at 5 ml., 5.5 ml., and 7.5 ml. measured to top of meniscus. Gum rubber stoppers to fit.

* Neutral butanol "special for milk work" is obtainable directly from Commercial Solvents Corporation, 17 East 42nd Street, New York, N. Y., and The Applied Research Institute, 15 West 34th Street, New York, N. Y.

Medicine droppers for use as pipettes.

Small ($\frac{1}{4}$ oz.) brown glass pharmaceutical dropping bottle with dropper calibrated to deliver 50 drops per ml. of BQC solution.

C. DETERMINATION

To 5 ml. of buffered substrate, add 0.5 ml. of milk sample. Shake briefly. Incubate for 10 minutes in a water bath at 36° to 44° C. If no water bath be available, incubate in pocket for a 15 to 20 minute period. Discontinue incubation, add 6 drops BQC solution. Shake well immediately. Allow to stand for 5 minutes. Appearance of blue color indicates inadequate pasteurization. For greater sensitivity add 2 ml. neutral n-butyl alcohol. Extract indophenol by completely inverting tube ten times, pausing after each 180° arc to allow bubbles to break and alcohol to separate. Rapid inversion will result in an emulsion, but if correctly performed, a clear supernatant alcohol layer. $\frac{3}{4}$ inch in height will result. Compare with color standards for field test using recommended filter.

D. INTERPRETATION

A value equal to or greater than 2 units generally indicates faulty pasteurization. See III, 1, G, p. 274.

E. CAUTION

All equipment should be thoroughly washed and rinsed before re-use. Avoid the use of phenolic resin bottle closures anywhere in the test. The BQC reagent is sufficiently sensitive to demonstrate the leaching of phenol from the resin by water.

Both solutions decompose with age and should be stored under refrigeration or prepared shortly before use.

A reagent blank should be made by adding 3 drops of BQC to 5 ml. of the substrate. If a blue color results, the substrate solution should be discarded. If the butyl alcohol procedure is utilized this reagent blank should be extracted with the alcohol.

METHOD NO. IV

*Method Developed by the Health Bureau Laboratories,
Rochester, N. Y.⁷*

A. COLLECTION OF SAMPLE

In the case of bottled milk, collect one or more bottles as prepared for sale. In the case of bulk milk, thoroughly mix by stirring with a suitable appliance long enough to reach to the bottom of the container. Continue mixing until all cream is evenly emulsified throughout the liquid.

Place samples in non-absorbent, airtight container and keep them refrigerated, but at a temperature above freezing, until ready for examination. No preservative should be added, and the sample should not be more than 2 days old.

B. REAGENTS

a. *Buffer substrate*—Dissolve 1.09 gm. of disodium phenyl phosphate and 11.54 gm. of sodium veronal (sodium diethyl barbiturate) in water and dilute to 1 liter. Add from 2 to 5 ml. of CHCl_3 per liter, and store in the refrigerator.

b. *Gibbs phenol reagent. 2, 6-dibromoquinonechloroimide solution*—Dissolve 0.2 gm. of the pure salt in 50 ml. of 95 per cent $\text{C}_2\text{H}_5\text{OH}$, place in a glass stoppered bottle, and store in the refrigerator. The solution should be discarded after storage for 1 week.

c. *Standard phenol solution*—Dissolve slightly more than 1 gm. of pure phenol in water, and standardize by the procedure outlined in the *U. S. Pharmacopocia XI*, p. 285, as follows: Dissolve about 1.5 gm. of phenol, accurately weighed, in sufficient distilled water to make 1,000 ml. of solution. Transfer an aliquot part of the solution, containing from 0.038 to 0.041 gm. of phenol, to a 500 ml. glass-stoppered flask having a long, narrow neck, add 30 ml. of tenth-normal bromine, then 5 ml. of hydrochloric acid, and immediately insert the stopper. Shake the flask repeatedly during $\frac{1}{2}$ hour, allow it to stand for 15 minutes, remove the stopper just sufficiently to introduce quickly 5 ml. of an aqueous solution of potassium iodide (1 in 5), being careful that no bromine vapor escapes, and at once stopper the flask. Shake the latter thoroughly, remove the stopper, and rinse

it and the neck of the flask with a little distilled water, so that the washings may flow into the flask, then add 1 ml. of chloroform, shake the mixture well, and titrate the liberated iodine with tenth-normal sodium thiosulfate, using starch T.S. as the indicator. Each ml. of tenth-normal bromine is equivalent to 0.001568 gm. of C_6H_5OH . Dilute the required amount of this solution to 100 ml. to give a solution containing 0.10 mg. of phenol per ml.

d. *Chloroform.*

C. APPARATUS

Test tubes

Pipettes, 1 ml. and 0.1 ml.

Incubator or water bath set at 37° C.

D. PROCEDURE

Add 1 ml. of sample of milk to 10 ml. of the buffer substrate solution, add a drop of $CHCl_3$, mix thoroughly by rotating the tube, and incubate at 37° C. for not less than 12 and not more than 18 hours. After incubation, add 0.1 ml. of the 2, 6-dibromoquinone-chloroimide solution and mix immediately. Compare the color with colors obtained in the controls of boiled milk, and boiled milk containing 0.01 mg. of phenol per ml.

E. INTERPRETATION

The absence of a blue color indicates that the sample was heated to 143° F. for 30 minutes. A color of the same or greater intensity than that of the 0.01 mg. phenol control indicates inadequate pasteurization.

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